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(57) Abstract

Improved assay methods and kits are described for the detection of changes in levels of human troponins in a patient sample, which employ as calibrators either a stable aqueous, acid-dialyzed solution of a troponin protein, the solution having a pH between about 2 and about 5 or lyophilized compositions derived from the aqueous solutions. Also described are modified troponin proteins, fusion proteins and hetero-multimers formed of troponin proteins or functional fragments.

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# STABILIZED PREPARATIONS OF HUMAN TROPONINS AND MODIFICATIONS THEREOF, DIAGNOSTIC ASSAY METHODS AND ASSAY KITS

## Field of the Invention

The invention relates generally to human and mammalian troponin proteins and assay methods and components employing them. More specifically, the invention provides stable aqueous and lyophilized preparations of troponin proteins characterized by low pH, as well as modified troponin proteins, fusion proteins and complexes, which are suitable for use as a calibrator/control standards in diagnostic assays for the detection of disorders or diseases characterized by damage to heart or skeletal muscle and for other research uses.

## Background of the Invention

Troponins are proteins located on the actin thin filament of vertebrate skeletal and cardiac muscles. Troponin is a complex of three subunits: troponin-C (TnC) is the calcium binding component; troponin-I (TnI) is the inhibitory subunit; and troponin-T is the protein which locates the complex on the tropomyosin complex. Cardiac and skeletal isoforms of TnI are similar in their sequences. The cardiac isoform differs substantially from its skeletal counterpart in possessing a 30-33 amino acid, species dependent N terminal extension. Previously these proteins were purified from heart or skeletal muscle. However, recently, genes for cardiac isoforms of TnI, TnC and TnT have been cloned and sequenced [Armour, K.L. *et al*, **1993**, Gene, 131:287-292; Vallins *et al*, **1990**, FEBS Lett., 270:57-61; R. Gahlman *et al*, **1988**, J. Mol. Biol.,

201:379-391, and Anderson, P. *et al*, **1995**, Circul. Res., 76:681-684]. Troponin proteins have previously been cloned, expressed and purified from an E. coli expression vectors [see, e.g., Al-Hillawi, E. *et al*, **1994**, Eur. J. Biochem., 225:1195-1201].

The troponin proteins have been proposed as biochemical markers for diseases and disorders of the heart and skeletal muscles, because these proteins alone or in complex are released into the plasma when the cardiac or skeletal muscles are damaged, such as in acute myocardial infarction, among other diseases. In fact, such proteins have been proposed to replace the present serum biochemical marker of choice for the diagnosis of AMI, the MB isoform of creatine Kinase [See, e.g., Adams, J.E. *et al*, **1993**, Circul., 88:750-763; Mangano, D. T., **1994**, Anesthes., 81(6):1317-1320; Bhayana, V. *et al*, **1995**, Clin. Chem., 41:312-317, among others].

A variety of enzymatic assays, immunoassays or radioassays have been proposed and developed for detection of troponins in patient samples. See, for example, the assays described in Larue, C. *et al*, **1993**, Clin. Chem., 39: 972-979; Styba *et al*, **1995**, Abstracts for Amer. Assoc. for Clin. Chem.; Wu *et al*, **1994**, Clin. Chem., 40(6):900-907; Muller-Bardorff *et al*, **1997**, Clin. Chem., 43(3):458-466; and Severina, M. *et al*, **1995**, Clin. Chem., 41, suppl. S151.

A limitation on the use and performance of such assays in clinical settings has been the stability of the preparations of the troponin proteins, used as standards or controls to calibrate the patient experimental results. Such standards for convenient use by clinical laboratories must be able to withstand a variety of storage and shipment conditions. For example, the frequently used

tissue derived human cardiac troponin I used in *in vitro* assays is insoluble in low ionic strength solutions and contains two cysteine residues which are extremely susceptible to oxidation. Recombinant HcTnI has been reported to be extremely susceptible to proteolysis [Hayden *et al*, 1995, Prot. Expr. Purif., 6:256-264]. This susceptibility to proteolysis has been a limitation to the use of HcTnI as a standard and/or calibrator in clinical assays. Furthermore, the proteins extreme sensitivity to proteolysis also makes stability an important issue. Buffered aqueous solutions of cTnI are stable for months at -80°C; however, this temperature requirement is not feasible for routine clinical laboratory use.

Even in lyophilized form, reconstitution of TnI has been shown to require the presence of at least 0.5M salt and/or a denaturant such as urea at a concentration of at least 6M to maintain solubility. This complicates the lyophilization and reconstitution of such a solution. A single report of dialysis of recombinant human cTnI followed by lyophilization [Al-Hillawi *et al.*, cited above] provided no teachings regarding the conditions under which the preparation was reconstituted for use, nor the stability of this lyophilized cTnI.

The search for a stabilized preparation of troponins has been avid, as revealed by such publications as UK Patent Application No. 2,275,774, published September 7, 1994; European patent application No. 743,522, published November 20, 1996; International application No. WO96/33415, published October 24, 1996; Canadian patent application No. 2,130,280, published February 25, 1995; and United States Patent No. 5,560,937 filed August 24, 1993. These reports, including product brochures from manufacturers of troponin preparations for use in assays,

require buffers at high pH and contain high concentrations of salts and/or urea.

As another example, fusion proteins containing HcTnI as part of the molecule have been designed. However, Armour *et al* fused HcTnI to ß-galactosidase in an effort to increase expression of the troponin in a bacterial system. Hayden *et al*, cited above, evaluated the solubility properties of an HcTnI-CKS fusion protein, but found no increase in the solubility of the fusion protein, but rather aggregates of the fusion protein in the initial crude extracts from bacterial culture.

There remains, therefore, a need in the art for compositions including stable and soluble troponin preparations for use as calibrators and controls in clinical assays for troponin levels in patients. Desirably such compositions would be stable over conventional conditions of storage and transport.

## Summary of the Invention

As one aspect, the invention provides an assay for measuring the level of a mammalian, preferably human, troponin protein in a patient sample. The assay includes the step of comparing the level in the sample with a novel troponin protein standard. In one embodiment of this aspect, the novel standard is a stable aqueous, acid-dialyzed solution of a troponin protein, the solution having a pH between about 2 and about 5. In a second embodiment of this aspect, the standard is a stable dry composition of a lyophilized acid-dialyzed solution of a troponin protein, the solution having a pH between about 2 and about 5. Surprisingly, the standard is reconstitutable to a stable liquid form by the addition of water, without the addition of any salt.

As another aspect, the invention provides an assay kit for measuring the level of a mammalian, preferably human, troponin protein in a patient sample. In addition to conventional assay reagents, this kit contains as its human troponin protein standard or calibrator a novel composition. In one embodiment, the novel composition is a stable aqueous, acid-dialyzed solution of a troponin protein, the solution having a pH between about 2 and about 5. In another embodiment, the novel calibrator is a stable dry composition comprising the lyophilized acid-dialyzed solution of a troponin protein, the solution having a pH between about 2 and about 5, the standard reconstitutable to a stable liquid form by the addition of water.

In yet a further aspect, the invention provides a stable aqueous, acid-dialyzed solution of a mammalian troponin protein having a pH between about 2 and about 5. In this solution, the troponin protein may be a mammalian troponin isoform, or a functional fragment thereof, a modified troponin protein or functional fragment thereof containing, e.g., an amino or carboxy terminal modification; a troponin protein or functional fragment thereof fused at its amino or carboxy terminus to a selected peptide or protein; a heterodimeric troponin complex or a heterotrimeric troponin complex.

In yet a further aspect, the invention provides a stable liquid composition suitable for assay calibrations comprising the solutions described above and a protein-based matrix comprising plasma components and stabilizers.

In still another aspect, the invention provides a lyophilized dry composition formed from the acid-dialyzed solutions described above, including that containing a matrix, the composition reconstitutable to a stable liquid

form by the addition of water and in the absence of salt.

Another aspect of the invention is a modified troponin protein which is a full-length or functional fragment of the troponin comprising at its amino or carboxy terminus a selected peptide, the modified protein having a pI lower than of an unmodified troponin protein. The modified troponins of this invention may be dialyzed or lyophilized as described above.

In still a further aspect, the invention provides a troponin fusion protein comprising a full-length or functional fragment of a troponin protein fused at its amino or carboxy terminus to a selected protein partner, the fusion protein having a pI lower than that of an unfused troponin protein. These fusion proteins may be dialyzed or lyophilized as described above.

In yet a further aspect, the invention provides a hetero-multimeric troponin protein complex. This complex may be dialyzed or lyophilized as described above. The complex may contain individual mammalian troponin proteins or functional fragments which are recombinant or native troponins, cardiac or skeletal troponins, and the members of the complex may originate from different mammalian species or different tissues from the same or different mammalian species. Similarly, each member of the complex may be a different isoform of the same troponin, or modified troponins or fusion proteins. The complex may be a heterodimer or a heterotrimer. The complex may be dialyzed or lyophilized as described above.

In another aspect, the invention provides a process for producing a stable aqueous solution of a human troponin protein. The process includes first dialyzing the protein against a suitable acid in a concentration sufficient to provide an acid/protein solution with a pH

between about 2 and about 5. In this case, the troponin protein may be a mammalian troponin, a functional fragment of a mammalian troponin, a modified troponin protein or functional fragment thereof as described above, a troponin fusion protein as described above or a hetero-multimeric troponin complex as described above.

In still another aspect, the invention provides a process for producing a water-reconstitutable, lyophilized composition comprising a mammalian troponin protein. This process includes the dialyzing step of the preceding process and a lyophilizing step, which results in a lyophilized composition which is reconstitutable in water in the absence of salt.

Other aspects and advantages of the present invention are described further in the following detailed description of the present invention.

Brief Description of the Drawings

Fig. 1 is a photograph of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrating the banding patterns of human cardiac troponin I, Lane 1; human cardiac troponin I containing a carboxy terminal 5 histidine-6 lysine-1 aspartate modified tail, (HcTnI-$K_6$-$H_5$-D) Lane 2; human cardiac troponin I-parvalbumin fusion protein (HcTnI-Pv) Lane 3; and human cardiac troponin I-human cardiac troponin C fusion protein (HcTnI-HcTnC) Lane 4. Each of the proteins were in the solutions defined by the final purification step as described in Example 1 for each protein respectively. All proteins have been electrophoresed in the absence of the reducing agent ß-mercaptoethanol in the sample buffer. The larger molecular weight bands in lanes 1, 2, and 4 represent dimeric forms of the molecules produced by the

oxidation of sulfydryl groups present in the molecules. Lane 3 demonstrates the lack of oxidation of these groups in the troponin I-parvalbumin construct by the absence of the higher molecular weight dimeric form.

Fig. 2 is a photograph of an SDS-PAGE gel illustrating the banding patterns of bacterial lysates of cultures co-expressing HcTnC and HcTnI. Lanes 1 and 2 are lysates of bacteria expressing troponin I and C together from the same plasmid. Lane 3 is a lysate of the BL-21 (DE3)pLysS host bacteria that do not contain a plasmid as a comparison to the expressing host lysates in lanes 1 and 2. The bands representing troponin I and troponin C are indicated in the figure as TnI and TnC respectively.

Fig. 3 is a photograph of an SDS-PAGE gel demonstrating the co-elution of HcTnI and HcTnC from DEAE Sephacel. The co-elution demonstrates the formation of a complex of the two troponin subunits, as HcTnI will not bind to the anion exchanger DEAE as an isolated subunit but will only do so when complexed with TnC. Lane 1, PL, is the sample before loading on the column. Lane 2, FT, is the flow-through. Lane 3, W, is the column wash. Lane 4, STD, is the HcTnI, HcTnC standard. Lane 5 is fraction 20 from the column. Subsequent lanes are labeled with the appropriate fraction number. Lanes labeled F25 and F30 show the correct stoichiometry for the two subunits when complexed as visualized on Coomassie blue stained SDS gels. The next lane marked F35 shows the elution of some complex along with excess free HcTnC (uncomplexed).

Fig. 4 is a photograph of a Western blot confirming the presence of HcTnI in fractions 25-45. Lanes correspond to the lanes in the SDS-PAGE gel of Fig. 3: lane 1 is flow through, lane 2 is wash, lane 3 is TnI standard, lanes 4-10 are fractions 18, 24, 30, 35, 40, 45

and 50, respectively. Primary antibody was a monoclonal antibody (mAb) raised against recombinant HcTnI designated 2A7-1E7, subclass $IgG_{2a}$. See Example 13.

Fig. 5 is a photograph of an SDS-PAGE gel demonstrating the integrity of isoform 3 of HcTnT ($HcTnT_3$) at three temperatures and as liquid or lyophilized samples. All samples were dialyzed, according to the invention, before storage. Lane 1 is a sample of a previous lot of recombinant HcTnT stored at -20°C shown as a comparison for the lot used for the stability study. All subsequent samples were from the same lot and stored at their respective temperatures and conditions for at least 30 days. Lane 2 is HcTnT stored as a liquid at ambient temperature; Lane 3 is HcTNT stored as a lyophilized powder at ambient temperature and reconstituted in the same volume of distilled water just prior to running the gel. Lane 4 is HcTnT stored as a liquid at 4°C; Lane 5 is HcTnT stored as a lyophilized powder at 4°C and reconstituted as above. Lane 6 is HcTnT stored as a liquid at 20°C. Lane 7 is HcTnT stored as a lyophilized powder at -20°C and reconstituted as above. Lane 8 is an untreated aliquot of HcTnT. Lane 9 illustrates molecular weight markers of 107,000, 76,000, 52,000, 36,800, 27,200 and 19,000 daltons. See Example 11.

Fig. 6 is a Western blot demonstrating the presence of HcTnC, HcTnI and HcTnT expressed from the same plasmid. A commercially available antibody against TnC, 1A7 (Biodesign International, ME), the mAb 2A7-1E7, and a goat polyclonal antibody raised against bovine cardiac TnT were used simultaneously as primary antibody. Lane 1: large culture of troponin complex (Tn) expressed in BL-21 (DE3)pLysS host; Lane 2: large culture of Tn expression in

BL-21(DE3); Lane 3: small culture of Tn expressed in BL-21(DE3); Lane 4: HcTnT standard; Lane 5: prestained molecular weight standards.

Fig. 7 is a photograph of an SDS-PAGE gel showing the presence of all troponin subunits after dialysis against either 1mM HCl according to this invention or against 5 mM ammonium bicarbonate. Lane 1: HcTnI/HcTnC complex dialyzed against ammonium bicarbonate; Lane 2: HcTnT/HcTnI/HcTnC complex dialyzed against ammonium bicarbonate; Lane 3: TnI/TnC complex dialyzed against HCl; Lane 4: TnT/TnI/TnC complex dialyzed against HCl; Lane 5: spill over, no sample; Lane 6: TnT/TnI/TnC standard; Lane 7: TnC standard; Lane 8: TnI standard; Lanes 9 and 10: TnT standards.

Fig. 8 is a photograph of an SDS-PAGE gel showing the stability of the troponin complexes after storage. Lanes 1-4 represent samples stored as lyophilized complexes. Lane 1: HcTnI/HcTnC complex dialyzed against ammonium bicarbonate and lyophilized for storage. Lane 2: TnI/TnC complex dialyzed against HCl according to the invention and lyophilized for storage. Lane 3: HcTnT/HcTnI/HcTnC complex dialyzed against ammonium bicarbonate and lyophilized for storage. Lane 4: TnT/TnI/TnC complex dialyzed against HCl according to this invention and lyophilized. Lane 5: TnT/TnI/TnC standard. Lane 6: TnT standard; Lane 7: TnI/TnC complex dialyzed against ammonium bicarbonate and kept as liquid for storage; Lane 8: TnI/TnC complex dialyzed against HCl according to this invention and kept as liquid for storage; Lane 9: TnT/TnI/TnC complex dialyzed against ammonium bicarbonate and kept as liquid for storage; Lane 10: TnT/TnI/TnC complex dialyzed against HCl according to this invention and kept as liquid for storage.

## Detailed Description of the Invention

The present invention meets the needs in the art for soluble compositions of mammalian, preferably human, troponin molecules which are stable under a variety of conditions on storage. Stable liquid solutions of substantially pure, mammalian troponins are provided by this invention, as well as stable lyophilized compositions of troponin which may be readily reconstituted in aqueous medium in the absence of urea or salt. The invention also provides methods of employing these stable solutions and preparations of troponins in assays formats, preferably immunoassays for the diagnosis and detection of damage to heart and skeletal muscle. As used herein, the term "stable" means that the solution or composition retains substantially all of its activity or immunogenicity over a wide range of temperature conditions and for up to about 60 days.

*I. Stabilized Liquid Solutions of Troponins*

In one embodiment, the invention provides a stable aqueous, acid-dialyzed solution of a mammalian troponin protein having a pH between about 2 and about 5. These low ionic strength troponin solutions are unexpectedly soluble in the absence of the urea or high salt concentrations considered necessary by the prior art to solubilize troponin protein solutions. In fact, the aqueous acid-protein solutions of the invention contain no substantial amounts of urea or salt, because these compounds are removed during dialysis. The solutions of this invention are stable under conventional reagent storage temperatures of between about 25 °C to about -80°C. The solutions are stable under such temperatures for periods of up to 60 days.

The stable, low ionic solutions of troponin proteins according to this invention are desirably prepared by dialyzing a selected troponin protein against a suitable acid in a concentration sufficient to provide the resulting aqueous acid/protein solution with a pH between about 2 and about 5. More desirably the pH of the resulting aqueous acid-protein is between about 2 and about 4; and most desirably, a pH of about 3 or 4. The conditions of dialysis can be variable depending upon the number and frequency of changes in dialysis solutions. As disclosed in detail below, in one example the solution was dialyzed four times against four liters of 1mM HCl at 4°C over two days with a change in solution every six hours. However, such conditions which are sufficient to acheive exchange of the contaminants and salts in solutions for the acid may be readily selected by those of skill in the art, and do not limit the practice of this invention.

Preferred in the method of preparing the solutions of this invention is the use of hydrochloric acid. However, in order to achieve the solutions of this invention, other strong acids such as sulfuric acid, hydrofluoric acid, nitric acid and phosphoric acid may be used in concentrations which will achieve the desired pH. In a particularly preferred example of this invention, a troponin solution of this invention is prepared by dialyzing the selected troponin protein with hydrochloric acid in a concentration of 1 mM, resulting in a pH of 3. Human cardiac troponin I is particularly useful in such a stable, soluble low ionic solution.

In still a further embellishment on these stable troponin solutions, the solutions may be mixed into another stable liquid composition suitable for immunoassay calibrations or preferably for use as controls in certain

assays, by mixing the solutions described above with protein-based matrices comprising known and commercially available plasma components and stabilizers.

The troponin proteins which may be contained in the stable solutions described above include naturally-occurring or recombinant troponin proteins alone, as well as naturally occurring or recombinant troponin proteins in modified form, or in complexes with other troponin proteins. For example, solutions according to this aspect of the invention include dialyzed solutions of mammalian, preferably human, troponin proteins which are naturally occurring and are isolated from a selected tissue, such as cardiac tissue or skeletal tissue. Alternatively, recombinant troponin proteins or functional fragments of such proteins may be stabilized into solutions according to this invention. By the term "functional fragment" as used herein means a portion of the complete protein sequence of the troponin molecule which portion retains the immunogenicity and immunoreactivity of the complete native protein. Solutions of this invention include, e.g., cardiac or skeletal troponin I, troponin C or troponin T, various isoforms of such proteins, or functional fragments thereof.

However, among other dialyzed solutions according to this invention are modified versions of a troponin protein or functional fragment thereof. Preferred modified troponins are modified troponin proteins having a pI lower than that of said unmodified troponin protein. Modified troponins may contain a carboxy terminal modification, including troponin proteins or functional fragments thereof which are fused at the carboxy terminus to a selected peptide or protein. Alternatively, similar modifications may be made to the

amino terminus of the selected protein. Such modified troponin molecules are described in detail below. These low ionic strength, stable and soluble solutions of the invention may also contain a heterodimeric troponin complex or a heterotrimeric troponin complex formed by, e.g., the association of troponins T, I and C, or a complex in which each troponin protein or functional fragment in said multimer differs from the other members of the multimer by tissue source, species origin, or isoform. Such multimers are also described in detail below.

As illustrated in the Examples below, illustrative dialyzed troponin solutions of this invention are soluble, stable on storage, retain the immunoreactivity of the native troponin molecule (e.g., HcTnI) by reactivity with antibodies raised to native HcTnI. Example 6 below specifically demonstrates that troponin protein solutions dialyzed against 1mM HCl from high salt and urea solutions according to this invention unexpectedly show no loss of solubility as demonstrated by a lack of precipitate present in the dialysate. Example 6 also compares total protein before and after dialysis according to this invention by densitometry on SDS-PAGE gels to demonstrate the stability of the solutions of this invention.

*II. Lyophilized Compositions of Troponins*

In another aspect and embodiment of the invention, the dialyzed troponin protein solutions described above may also be provided in a dry composition. The dialyzed solutions of the invention may be lyophilized, or freeze-dried, by conventional methods. As described above, prior to lyophilization the selected troponin compound is dialyzed against an acidic solution

to produce a low ionic strength solution consisting essentially of the protein without any substantial amounts of either urea or salt. Among other things, the dialysis step removes salts and other materials (i.e. urea) that can crystallize or otherwise interfere with lyophilization of the protein. The lyophilized compositions are stable on storage in the lyophilized form.

Surprisingly, once lyophilized by conventional techniques, the dry troponin composition may be reconstituted in an aqueous medium, preferably distilled water, also without the addition of urea or high salt, previously believed essential to maintaining the solubility of a troponin protein. The reconstituted troponin protein compositions are stable on storage and, when reconstituted in water, demonstrate substantially no loss of function in an immunoassay.

As illustrated in the examples below, the dialyzed troponin protein solutions of the invention may be lyophilized and stored under various conditions. For example, dialyzed, lyophilized troponin compositions of the invention proteins are stored at 25° to -80°C, or at room temperature, and completely reconstituted in distilled water to a low ionic strength solution. The lyophilized compositions have been shown to be stable over time under the described conditions. The examples further demonstrate that no loss of protein occurs during reconstitution, demonstrating complete solubility under the described conditions. As further demonstrated in Example 6, all of the troponin proteins were able to be lyophilized and reconstituted with minimal loss of protein concentration as judged on SDS-PAGE gels either visually or by densitometry. The lyophilized and reconstituted troponin molecules have not lost the immunogenicity of the

native molecule. For example, immunoreactivities of lyophilized troponin preparations are compared with that of native human cardiac troponin I using a specific monoclonal antibody and a conventional ELISA in Example 6 and the immunoreactivities of the compositions of this invention are found to be identical with that of the native protein.

*III. Modified Troponins*

Still a further embodiment of the present invention is provided by modifications of the troponin proteins which enhance the solubility and stability of the compound.

According to this invention, a modified troponin protein is a full-length or functional fragment of a troponin having an increased polarity compared to that of the unmodified troponin.

One type of modified troponin according to this invention bears on its carboxy terminus a peptide, which is a sequence of amino acid residues having a higher polarity that than of unmodified troponin. Another such modified troponin has a similar modification on its amino terminus. It is readily known to those of skill in the art which amino acids have high polarities, e.g., including *inter alia*, histidine, asparatic acid. One of skill in the art may readily select other known amino acids with high polarities to design a peptide having a desired high polarity to enable the modified troponin to achieve an increased polarity in comparison to the unmodified troponin. Desirably, such peptides are of between about 5 and about 30 amino acids in length, although they may be larger, if desired.

Another modified troponin, which is stable under a variety of storage conditions, even when the modified

troponin is not in a stable dialyzed solution or lyophilized composition described above is a troponin modified by adding to the carboxy terminus of the protein a sequence of 6 lysines, 5 histidines and 1 aspartic acid. See, particularly, Example 2, which teaches HcTnI-$K_6$-$H_5$-D.

Still another particular example of a stable carboxy terminal modification of a troponin molecule is prepared by adding to the carboxy terminus of the protein a sequence comprised of three alternating histidines and three alternating leucines. See, Example 3, which teaches HcTnI-$(HL)_3$.

Any number of similar peptides may be used to modify a troponin (naturally occurring, isolated from tissue or recombinant) to have an increased polarity or to modify the troponin at the carboxy or amino terminus. Such peptides may be designed by one of skill in the art from among known amino acids with high polarities and introduced onto the amino or carboxy terminus of mammalian, preferably human, troponins e.g., TnI, TnC or TnT, by recombinant techniques or by chemical cross-linking methods known to those of skill in the art. The troponins so modified may be derived from a variety of mammalian tissues, or prepared recombinantly. Similarly, such modified troponins may also be dialyzed into stable solutions as described above, and/or lyophilized as described above.

A second type of modified troponin useful according to the present invention is a troponin fusion protein which is made up of a full-length or functional fragment of a selected troponin protein fused at its carboxy or amino terminus to a selected protein partner, the resulting fusion protein having a pI lower than that of the unfused troponin protein. Unfused or unmodifed

troponin has a pI of about 7-8. As with the peptide modification described above, one such fusion protein desirably has the selected troponin, i.e., TnI, TnC or TnT, as the N terminal protein, fused to a second protein having a net negative charge sufficient when fused to the troponin protein to achieve the desired pI.

The selection of the carboxy terminal fusion protein or amino terminal fusion protein having a suitable net negative charge or low ionic strength is well within the skill of the art. The identity of that fusion partner protein is not a limitation upon this modification. The fusion partner may be fused in frame directly to the N terminal troponin molecule, or it may be fused to the N terminal protein by means of an optional and conventional linker or spacer sequence.

Two examples are provided below. As one example of a suitable fusion partner, the protein is parvalbumin. Other non-interacting proteins having low pIs may also be employed as fusion partners. As demonstrated in Example 3 below, a fusion protein made of HcTnI fused to carp parvalbumin is constructed by resort to conventional genetic engineering techniques. However, where the troponin is a native protein isolated or purified from tissue, the attachment of the fusion partner may be by conventional chemical cross-linking methods.

The resulting HcTnI-Pv fusion protein has an additional quality of being less susceptible to the formation of interchain disulfide bonds due to oxidation than the other TnI's as seen on SDS-PAGE run under non-reducing conditions therefore increasing the stability of this modified protein (see Fig. 1).

As another example of a fusion partner, the

carboxy or amino protein partner may be another troponin protein or fragment thereof. Such a troponin fusion partner may be derived from a different species of mammal than the first troponin protein. It may be a different isoform of the same troponin protein. It may be derived from a different tissue source than the first protein. As one example, the N terminal protein is HcTnI, and the carboxy terminal fusion partner is HcTnC or a functional fragment thereof. See, for example, the fusion protein of Example 4.

Any number of similar fusion proteins may be designed by one of skill in the art by selecting as the fusion partner protein, a protein from among known proteins with low ionic strengths, and introducing it onto the amino or carboxy terminal of a troponins TnI, TnC or TnT, or fragment thereof to generate modified troponins having the appropriate low pI.

Fusion or linkage between the troponin protein and the carboxy terminal fusion partner may be by any suitable means, e.g., by conventional covalent or ionic bonds, protein fusions, or hetero-bifunctional cross-linkers, e.g., carbodiimide, glutaraldehyde, and the like. Such techniques are known in the art and readily described in conventional chemistry and biochemistry texts.

Additionally, conventional linker or spacer sequences which simply provide for a desired amount of space between the second protein partner and the troponin protein may also be constructed into the modified fusion protein. The design of such linkers is well known to those of skill in the art.

Such modified troponin fusion proteins may also be dialyzed into stable solutions as described above, and/or lyophilized as described above.

*IV. Troponin Complexes*

As yet a further aspect of this invention, the troponin proteins may be assembled into a hetero-multimeric troponin protein complex. Each member of the multimer is selected from a troponin protein or functional fragment thereof which differs from the other members of the multimer by mammalian species origin, by tissue source, by isoform or by method of production. For example, one such multimer may be formed of a recombinant human cardiac troponin I complexed to a recombinant skeletal troponin T, and recombinant human cardiac troponin C. Similarly the complexed troponins may be recombinant or native proteins, wildtype or modified proteins. They can be formed *in vitro* by mixing the appropriate amounts of the members of the troponins under suitable conditions [see, e.g., Potter, cited above]. The troponins of the multimer may be expressed recombinantly in a host cell.

The multimer can be a heterodimer, formed, for instance, of the assembly of TnC complexed to a TnI. Another desirable dimer is the complex formed by a TnC complexed to a TnT. Still another desirable dimer is formed by a TnI complexed to a TnT. In the formation of these dimeric complexes, it is not critical which troponin is listed first, because the assembly can occur naturally within a transfected host cell. The multimer can be a heterotrimer of TnC, TnI and TnT, in the three dimensional structure dictated by their assembly in a host cell. Complexes formed by functional fragments of these troponins are also included. Specific linear sequences of the different troponins in any order may be produced by fusion as described above.

These multimeric complexes may be dialyzed and/or lyophilized as described above. It is anticipated that other troponins which may form complexes may also be used in the low ionic strength solutions of this invention.

According to one aspect of the invention, the assembly of troponin multimers is accomplished by expressing the gene encoding each troponin (or a functional fragment thereof) on a separate plasmid under the control of regulatory sequences directing the expression of the protein in a host cell. For example, in the construction of a heterodimer, the selected troponin genes on selected expression plasmids are co-transfected into a host cell. Once the host cell is cultured, the troponins are expressed and assemble within the cell. The cell is then lysed by conventional techniques and the complex isolated. In the case of the trimer, three separate plasmids may be designed and co-transfected into the same cell.

Alternatively, two or more of the genes encoding the troponins or functional fragments may be placed on the same plasmid under the control of the same, or different promoter regulators. Thus, the host cell may be transfected with a single plasmid containing all two or three of the troponins, and the culturing and isolation of the complex would occur in the manner described above.

These troponin complexes isolated from the host cells are stable, soluble molecules. This aspect of the invention provides a complex directly isolated from the host cell, therefore bypassing the need to form the complex from the isolated components *in vitro*.

*V. Methods of Preparing the Modified Proteins and Complexes*

The modified proteins described above, or the complexes described above, as well as the individual troponins of this invention can be expressed in recombinant host cells, e.g., mammalian, bacterial, fungal, insect, etc., by resort to recombinant DNA technology using genetic engineering techniques given the specific teachings of the invention provided herein. The same or similar techniques may also be employed to generate other embodiments of this invention.

Thus, a nucleotide molecule which contains the nucleotide sequence encoding a modified protein or complex described above, optionally under the control of regulatory sequences directing expression of the protein in a selected host cell may be designed. Briefly described, a conventional expression vector or recombinant plasmid is produced by placing coding sequences for the modified troponins or complexes in operative association with conventional regulatory control sequences capable of controlling the replication and expression in, and/or secretion from, a host cell.

Regulatory sequences include promoter sequences, e.g., CMV promoter, and optional signal sequences. The troponin molecules may be expressed individually under the control of individual regulatory sequences, or in tandem, as in known in the art. A selected host cell is transfected or co-transfected by conventional techniques with either a single vector expressing a single troponin or two or more troponions, or co-transfected with more than one plasmid vector to create the transfected host cell of the invention comprising the recombinant modified troponin or troponin complex. The transfected cell is then cultured by conventional techniques to produce the proteins or complex of the invention. The production of

the troponin complex which includes the association of individual troponins with each other is measured in the culture by an appropriate assay, such as ELISA or RIA. Similar conventional techniques may be employed to construct other molecules of this invention.

Suitable vectors for the cloning and subcloning steps employed in the methods and construction of the compositions of this invention may be selected by one of skill in the art. For example, the conventional pUC series of cloning vectors, may be used. One vector used is pUC19, which is commercially available from supply houses, such as Amersham (Buckinghamshire, United Kingdom) or Pharmacia (Uppsala, Sweden). Additionally, any vector which is capable of replicating readily, has an abundance of cloning sites and selectable genes (e.g., antibiotic resistance), and is easily manipulated, may be used for cloning. Thus, the selection of the cloning vector is not a limiting factor in this invention.

Similarly, the vectors employed for expression of the recombinant proteins, modified proteins and/or complexes according to this invention may be selected by one of skill in the art from any conventional vectors. See, for example, the vectors employed in the examples. The vectors also contain selected regulatory sequences (such as CMV promoters) which direct the replication and expression of heterologous DNA sequences in selected host cells. These vectors contain the above described DNA sequences which code for the troponin protein(s). In addition, the vectors may incorporate selected troponin sequences modified by the insertion of desirable restriction sites for ready manipulation.

The expression vectors may also be characterized by genes suitable for amplifying expression of the

heterologous DNA sequences, e.g., the mammalian dihydrofolate reductase gene (DHFR). Other preferable vector sequences include a polyadenylation (poly A) signal sequence, such as from bovine growth hormone (BGH) and the betaglobin promoter sequence (betaglopro). The expression vectors useful herein may be synthesized by techniques well known to those skilled in this art.

The components of such vectors, e.g. replicons, selection genes, enhancers, promoters, signal sequences and the like, may be obtained from commercial or natural sources or synthesized by known procedures for use in directing the expression and/or secretion of the product of the recombinant DNA in a selected host. Other appropriate expression vectors of which numerous types are known in the art for mammalian, bacterial, insect, yeast, and fungal expression may also be selected for this purpose.

The present invention also encompasses a cell transfected with a recombinant plasmid containing the coding sequences of the modified troponin molecules or complexes. Host cells useful for the cloning and other manipulations of these cloning vectors are also conventional. However, most desirably, cells from various strains of *E. coli* are used for replication of the cloning vectors and other steps in the construction of the recombinant proteins and complexes of this invention.

Suitable host cells or cell lines for the expression of the troponin proteins and complexes of the invention are preferably bacterial cells [see, e.g., Plückthun, A., **1992**, *Immunol. Rev.*, *130*:151-188]. The tendency of proteins expressed in bacterial cells to be in an unfolded or improperly folded form or in a non-glycosylated form does not pose as great a concern as

troponins are not normally glycosylated and can be engineered for exported expression thereby reducing the high concentration that facilitates misfolding. Nevertheless, any recombinant troponin produced in a bacterial cell would be screened for retention of immunoreactivity. If the molecule expressed by the bacterial cell was produced and exported in a properly folded form, that bacterial cell would be a desirable host. For example, various strains of *E. coli* used for expression are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Streptomyces*, other bacilli and the like may also be employed in this method. See, also the *E. coli* strain used in the following examples. Other *E. coli* expression systems are described in Studier, F.W. *et al*, **1990**, Meth. Enzymol., 185:60-89.

Also useful may be mammalian cells such as CHO, COS, a fibroblast cell (e.g., 3T3), and myeloid cells, and more preferably a CHO or a myeloid cell. Human cells may be used, thus enabling the molecule to be modified with human glycosylation patterns. Alternatively, other eukaryotic cell lines may be employed.

Where desired, strains of yeast cells known to those skilled in the art are also available as host cells, as well as insect cells, e.g. *Drosophila* and *Lepidoptera* and viral expression systems. See, e.g. Miller *et al.*, **1986**, *Genetic Engineering*, *8*:277-298 and references cited therein.

The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Sambrook *et al.*, **1989**, Molecular Cloning (A

Laboratory Manual), 2nd edit., Cold Spring Harbor Laboratory (New York).

The general methods by which the vectors of the invention may be constructed, the transfection methods required to produce the host cells of the invention, and culture methods necessary to produce the troponin molecules or complexes of the invention from such host cells are all conventional techniques. Likewise, once produced, the proteins or complexes of the invention may be purified from the cell culture contents according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Such techniques are within the skill of the art and do not limit this invention.

*VI. Assay Formats using same and kits; Other utilities*

The above described dialyzed solutions or lyophilized compositions containing the recombinant troponins, modified troponin proteins and/or complexes described above are useful as calibrators, controls or standards in diagnostic assays for diagnosis of damage to heart or skeletal muscle.

The stable solutions and/or lyophilized compositions of the inventions may be useful to replace the controls or standards in any assay format which requires a standard. Among such assays are immunoassays, such as described in Larue, C. *et al;* Styba *et al;* Wu *et al;* Muller-Bardorff *et al*, and Severina, M. *et al*, UK Patent Application No. 2,275,774; European patent application No. 743,522; International application No. WO96/33415; Canadian patent application No. 2,130,280 and

United States Patent No. 5,560,937, all cited above in the background.

In general, the compositions of this invention are useful in a variety of immunoassays for measuring the level, or detecting the presence or absence of a mammalian troponin protein in a patient sample. Simply put, the assay methods entail the use of antibodies to the selected troponin to be measured, and signal generating second antibodies or conventional signal systems to measure the level of the selected troponin in the patient's serum or plasma sample. The measured patient troponin level is then compared with a troponin protein standard and a determination is made whereby the patient's level is equivalent, absent, lower or elevated with respect to the standard. According to this invention, the standard in any suitable assay may be replaced with a stable aqueous, acid-dialyzed solution of a recombinant troponin protein as described above. In another embodiment, the standard may be replace with a stable lyophilized acid-dialyzed composition of a recombinant troponin protein, which lyophilized composition may be reconstituted to a stable liquid form by the addition of water alone. However, the nature of the assay does not limit the application of the compositions of this invention.

As another embodiment, the present invention provides a diagnostic kit which may be used in a clinical laboratory to aid in the diagnosis or assessment of the condition of a patient suspected of having a disorder resulting in cardiac or skeletal muscle damage. The methods and assay components described herein for the collection and measurement of the troponin in serum or plasma may be efficiently utilized in the assembly of such

a kit for the detection of troponin using the compositions of this invention as the standard.

Primarily, the kit according to this invention would contain a composition of this invention as the standard, control or calibrator, i.e., a stable aqueous, acid-dialyzed solution of a recombinant troponin protein, as described herein or a stable dry composition formed by lyophilized an acid-dialyzed solution of a recombinant troponin protein as described, or undialyzed modified troponins or troponin complexes of the invention. A suitable kit may also contain an appropriate binding ligand capable of binding the troponin, an appropriate assay indicator molecule, reverse phase cartridges, assay buffers, matrix buffers and other conventional elements. Such an appropriate binding ligand may be selected by one of skill in the art and may be, for example, an antibody, a receptor, or other conventional ligand. This kit may be employed for the performance of one or more assay methods. Components of the kit may vary according to the purposes of the assay.

Advantageously, use of such a kit, and the method of the invention provides an easy and accurate method for measuring troponin in serum or plasma. It is further anticipated that use of the multimeric complexes as the control or calibrator, may permit diagnostic evaluations even more accurate than those permitted by use of a single troponin protein as the calibrator. As described above with regard to the assays, the components of the kit and the type of assay for which it is intended is not a limitation of this invention, a kit for any assay employing a troponin standard may contain the compositions of this invention.

Still other utilities of the present invention include research uses, such as uses in methods for studying normal and pathological functions of the heart, skeletal muscle or any other tissue source from which a troponin is derived. Additionally, the compositions of this invention are useful in assays to determine the concentration of a troponin for other than diagnostic use, or for purification techniques, or to generate antibodies, all by conventional methods.

The following examples illustrate the method of preparing a stable formulation of a human troponin protein or fragment or modification thereof. These examples are illustrative only and do not limit the scope of the present invention. The following examples demonstrate human cardiac troponin I and modifications prepared according to embodiments of this invention. The HcTnI preparations provide a calibrator/control standard for use in assays for circulating cardiac troponin I and thus serve as an aid in the diagnosis of acute myocardial infarction and other related disorders. The following examples illustrate modifications to human cardiac TnI which include: wild type HcTnI dialyzed and lyophilized according to this invention; modified HcTnI with a C terminal addition of 6 lysines, 5 histidines and 1 aspartic acid (HcTnI-$K_6$-$H_5$-D); modified HcTnI with a C terminal addition of 3 leucines alternating with 3 histidines (HcTnI-$[LH]_3$); a fusion protein formed of HcTnI and a carboxy terminal fusion partner, carp parvalbumin; a fusion protein formed by HcTnI fused to human cardiac troponin C (HcTnI-HcTnC). Also illustrated is the recombinant expression of a composition according to this invention of the isoform 3 of HcTnT and its reactivity in

an immunoassay, as well as preparations of the complexes of this invention.

EXAMPLE 1: RECOMBINANT HUMAN CARDIAC TROPONIN I

Recombinant human cardiac troponin I (HcTnI) was made by extraction of total RNA from human heart following the method of Chomcynski, P., and Sacci, N., **1987**, Anal. Biochem., 162:156-159. RNA was transcribed into a cDNA using reverse transcriptase, cDNA Cycle Kit for RT-PCR (Invitrogen, San Diego, CA).

Using the published DNA sequence of HcTnI [Vallins *et al*, cited above; which is SEQ ID NO: 1 with the modification of a codon for Thr at amino acid position 86], a 3' oligonucleotide complementary to the 3' end of the coding region of the HcTnI gene was synthesized for use as the annealing primer for synthesis of the first strand of cDNA. The first strand of cDNA generated by reverse transcriptase was then used as a template for PCR amplification. The PCR reaction was primed using the 3' oligonucleotide and a 5' oligonucleotide synthesized to correspond to the 5' end of the coding region for HcTnI. Each primer contained a restriction site at its 5' end to facilitate subsequent subcloning.

The PCR cycles were as follows: 2 minutes at 94°C, 30 cycles of 30 seconds at 95°C, 2 minutes at 50°C, 2 minutes at 72°C with the last cycle ending in 10 minutes at 72°C. The PCR product was isolated on a low melt agarose gel, cut out and cleaned with gene clean (BIO 101, Vista, CA).

The product and prokaryotic plasmid vector pET 11d (Novagen, Madison, WI) containing a multiple cloning site were digested with restriction enzymes NcoI and BamHI (Gibco-BRL, Gaithersburg, MD). The two DNA strands were ligated using T4 DNA ligase (Boehringer Mannheim,

Indianapolis, IN). *E. coli* DH5 α, a high transformation efficiency host, was transformed with the insert-containing plasmid. DH5 α is used for transformations from ligation reactions because it is 10 times more efficient for transformation than BL21 and it is used for amplification of the DNA. This vector is not able to express the target protein however, and BL21 must be used with pET vectors for this purpose. However, other *E.coli* strains and other vectors serving the same purposes can also be used.

Colonies of DH5 α that contained the plasmid were selected by growth on LB agar containing ampicillin. The plasmid contains the sequence coding for ß lactamase production, thereby conferring resistance to ampicillin to the transformed bacteria. The plasmid DNA was isolated by standard alkaline lysis method and sequenced using the Sequenase DNA sequencing kit (United States Biochemical, Cleveland, OH). The sequence of the HcTnI cDNA was found to be identical to the published sequence for this molecule [Armour, K.L. *et al*, 1993, Gene, 131:287-292; SEQ ID NO: 1]). These sequences are also similar to that described in Vallins *et al.*, cited above, with the exception of one amino acid change at amino acid position 86.

*E. coli* BL21 (DE3), a preferred host for pET vectors, was transformed with the vector. The culture was grown n enriched media containing yeast extract, tryptone and M9 minimal salts (Sigma, St. Louis, MI) in the presence of 200 µg/ml ampicillin (plasmid resistance marker) to mid log phase and induced by making the culture 1 mM in isopropyl-B-D-thiogalactoside (IPTG; Boehringer Mannheim, Indianapolis, IN). Recombinant HcTnI was isolated from

the bacterial culture by sonication of the bacterial pellet in 6M urea, 10 mM sodium citrate, pH 5.0, 2 mM EDTA, 1mM dithiothreitol (DTT). The sonicate was centrifuged at 48,000 xg.

The supernatant was loaded on a CM-52 column equilibrated in the same buffer. The column was eluted with a 500:500 ml 0 - 0.5M NaCl gradient. The fractions containing HcTnI were pooled and dialyzed against 4 liters of 4M urea, 50 mM Tris, pH 7.5, 1M NaCl and 1 mM DTT, 4 liters of 2M urea, 50 mM Tris, pH 7.5, 1M NaCl and 1 mM DTT and finally 2 times 4 liters of 50 mM Tris, pH 7.5, 1M NaCl and 1 mM DTT. The dialyzed pool was loaded onto a TnC affinity column which consisted of cyanogen bromide activated sepharose 4B coupled with rabbit skeletal TnC as described [Potter, J.D, **1982**, Meth. Enzymol., 85:241-263]. The column was equilibrated in the same buffer before the protein was loaded. After the protein was bound the column was washed and eluted with a 125:125 ml gradient from 0M urea, 0M EDTA to 6M urea, 3mM EDTA. HcTnI was judged to be >95% pure based on observation of Coomassie blue stained protein bands on the SDS-PAGE gel of Fig. 1.

EXAMPLE 2: RECOMBINANT MODIFIED HcTnI WITH A CARBOXY TAIL

Recombinant HcTnI described in Example 1 above was modified to add six lysines, five histidines and one aspartate to its C-terminal end (HcTnI-$K_6$-$H_5$-D [SEQ ID NO: 5]). This modification was made to change the isoelectric point of the protein, thereby improving the solubility and stability of the resulting molecule.

The HcTnI cDNA described in Example 1 above [SEQ ID NO: 1] was used as a template for PCR. The cDNA encoding this protein was synthesized using PCR to add the C terminal modifications to the HcTnI. The PCR reaction was

primed using the 5' oligonucleotide used for the synthesis of HcTnI as described in Example 1 and a 3' primer having the sequence 5' GT GGATCC TCA GTG ATG GTG ATG GTG ATG TTT CTT TTT CTT TTT CTT GCT CTC AAA CTT TTT CTT GCG G 3' [SEQ ID NO: 3]. The sequence of this 3' oligo encodes reading from its 3' end contains a sequence complementary to the nucleotides 630-609 in the HcTnI sequence of SEQ ID NO: 1 (underlined), followed by the codons coding for 6 lysines and 6 histidines followed by a translational stop codon and a BamHI restriction site.

The PCR product was purified on an agarose gel, digested with BamHI and NcoI and ligated with a similarly digested pET 11d plasmid. Using the procedure as previously described in Example 1, the *E. coli* DH5 α was transformed with the ligated plasmid and colonies were selected by growth on LB agar containing ampicillin. The plasmid DNA was isolated from bacterial cultures and sequenced.

Although the 3' oligo was synthesized correctly, DNA sequencing revealed the C terminal amino acid to be aspartate rather than the expected histidine. Other than this one change, the sequence was found to be the desired sequence; HcTnI with 6 lysines, 5 histidines and one aspartate on the C terminus (HcTnI-$K_6$-$H_5$-D [SEQ ID NO: 5]). The expression host *E. coli* BL21 (DE3) was transformed with the plasmid vector. Bacterial cultures were grown in enriched media in the presence of 200 μg/ml ampicillin to mid log phase and induced by making the culture 1 mM in IPTG. The bacterial pellet was sonicated in 50 mM sodium phosphate, pH 8.0. 300 mM NaCl and 6M urea. The sonicate was centrifuged at 48,000 xg and the supernatant was applied to a $Ni^{2+}$ affinity (Qiagen, Chatsworth, CA) column equilibrated with the same buffer.

The column was washed with 50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 6M urea and 10% glycerol. The bound protein was eluted with a 100:100 ml gradient 0 - 0.4M imidazole. The modified HcTnI-$K_6$-$H_5$-D was pure as eluted from the $Ni^{2+}$ affinity column judged by SDS-PAGE.

The DNA sequence of this modification, HcTnI-$K_6$-$H_5$-D, is shown in SEQ ID NO. 4, and the deduced amino acid sequence is shown in SEQ. ID NO. 5.

EXAMPLE 3: HcTnI\HcTnC FUSION PROTEIN

HcTnI and HcTnC were expressed as a single protein with the N-terminal being TnI and the C-terminal being TnC (HcTnI-HcTnC fusion). The addition of the calcium binding protein HcTnC was made to provide more favorable solubility properties and to improve the solubility of the resulting fusion protein.

The cDNA encoding this protein was synthesized by a two step PCR procedure. Two PCR reactions were used to generate the intermediate cDNA's needed. In the first PCR reaction, the 5' primer encoded part of the plasmid vector upstream of the coding region; the 3' primer used was a synthesized oligonucleotide sequence: 5' CGCAGCCTTGTAGATGTCATCCATGCT CTCAAACTTTTTCTTGCGGCCCTC 3' [SEQ ID NO: 6]. This sequence is a complementary sequence encoding the C terminal eight amino acids of HcTnI followed by the N terminal eight amino acids of HcTnC and the template used was HcTnI plasmid DNA. The product of this PCR reaction was the DNA sequence encoding the entire amino acid sequence of HcTnI followed by the sequence coding for the first eight amino acids of HcTnC.

For the second PCR reaction, the 5' primer used was a synthesized oligonucleotide sequence: 5' AGGGCCGCAAGAAAAAG TTTGAGAGCATGGATGACATCTACAAGGCTGGCTGCGGTAG 3' [SEQ ID NO:

7]. The 3' primer was a complementary primer that encodes part of the plasmid vector downstream of the insert, and the template was HcTnC plasmid DNA. The cDNA coding for HcTnC was generated from RT-PCR as described for HcTnI above using the published sequence to synthesize appropriate 5' and 3' primers [Gahlmann, R. *et al*, cited above] and ligated into a plasmid vector. The product of the second PCR product was a sequence encoding the C terminal eight amino acids of HcTnI followed by the entire sequence coding for HcTnC.

Both PCR products were purified on an agarose gel and used as templates/primers for a third PCR reaction. This reaction also used as primers, a sequence of the plasmid upstream of the coding region and another complementary to a region downstream of the coding sequence. During the first cycle of PCR, after denaturing, some of the first and second PCR sequences act as primers and templates for each other, so that full length products coding for HcTnI followed by HcTnC are obtained in the first cycles of PCR.

This cDNA was restriction digested using BamHI and NcoI and ligated with a similarly digested pET 11d plasmid. DH5 $\alpha$ was transformed with the resulting vector and colonies selected by growth on LB agar containing ampicillin. The plasmid DNA was isolated from bacterial cultures and sequenced. The sequence was found to be as predicted, namely, the full sequence coding for HcTnI followed immediately by the full sequence coding for HcTnC.

The HTnI portion of the protein has the DNA sequence as shown in SEQ ID NO: 1 and the HTnC portion of the fusion protein has the DNA sequence as published in Gahlmann, R. *et al*, cited above, i.e., from nucleotide 631 to 1116 of SEQ ID NO:8. The plasmid was then used to

transform *E. coli* BL 21 (DE3). Cultures were grown in enriched media to mid log phase and induced with 1 mM IPTG. The bacterial pellet was sonicated in 50 mM Tris, pH 8.0, 5 mM EDTA, 0.1 mM DTT, 6M urea.

The supernatant was applied to a DE-52 column equilibrated with the same buffer. The column was washed and then eluted with a 500:500 ml gradient 0-0.6 M NaCl. The protein bound to DE-52 which is a characteristic of TnC but not TnI. It eluted at an ionic strength between 1.67 and 5.6 mS/cm. The fractions containing HcTnI-HcTnC fusion protein were pooled and the dialyzed pool was loaded on a S-Sepharose column equilibrated with he same buffer, washed and eluted with a 200:200 ml gradient 0.1-0.3M NaCl. The HcTnI-HcTnC fusion protein eluted at an ionic strength between 7.0 and 8.0 mS/cm. Some additional HcTnI-HcTnC fusion protein was eluted from the column with a 0.4 M NaCl step, ionic strength 14 mS/cm. This protein was judged to be pure by SDS-PAGE.

The DNA sequence encoding this fusion protein HcTnI-HcTnC, is shown in SEQ ID NO: 8 and the deduced amino acid sequence of the fusion protein as SEQ ID NO: 9. Nucleotide sequence positions #631-633 of SEQ ID NO: 8 of the wild type DNA sequence represents the translational stop codon. This codon terminates translation of the messenger into protein and cannot appear at this position in the fusion proteins or translation will be terminated before the full length protein is produced.

EXAMPLE 4: HcTnI-CARP PARVALBUMIN FUSION PROTEIN

HcTnI and carp parvalbumin were expressed as a single fusion protein with HcTnI being the N-terminal portion and carp parvalbumin (Pv) being the C-terminal portion (HcTnI-Pv) [see, e.g., Coffe, C.J. and Bradshaw, R.A., **1973**, J.

Biol. Chem., 248:3305-3312]. Carp parvalbumin is a calcium binding protein and, as in the HTnI-HTnC fusion protein, this modification was made to provide favorable solubility properties and to improve the solubility of the resulting fusion protein.

This cDNA was synthesized using a two step PCR procedure. The first PCR step used HcTnI plasmid DNA as the template. The 5' primer was the promoter region of the bacterial plasmid. The 3' primer was a synthesized oligonucleotide having the sequence 5' AGCGTCGTTCAGAAC ACCAGCGAAAGCCATGCTCTCAAACTTTTTCTTGCGGCCCTC 3' [SEQ ID NO: 10]. The PCR product sequence was that of HcTnI with the first 30 base pairs of parvalbumin on its 3' end.

The second PCR template was a carp parvalbumin insert in a bacterial plasmid vector DNA. The 5' primer used was a synthesized oligonucleotide having the sequence: 5' AGGGCCG CAAGAAAAAGTTTGAGAGCATGGCTTTCGCTGGTGTTCTGAACGACGCTG [SEQ ID NO: 11]. The 3' primer was complementary to a portion of the plasmid downstream of the multiple cloning site.

The resulting PCR product DNA sequence was that of carp parvalbumin with the last 26 base pairs of HcTnI on the 5' end. These two PCR products were used as primers and templates for each other as described for the construction of the HcTnI-HcTnC fusion protein in a third, overlap PCR reaction.

The product of this final PCR reaction was a nucleotide sequence encoding the complete HcTnI sequence of SEQ ID NO: 1 followed by the complete carp parvalbumin sequence, i.e., nucleotides 631-960 of SEQ ID NO: 12 and amino acid 191-299 of SEQ ID NO: 13. Both proteins were encoded as a single fusion product. The PCR DNA was restriction digested with NcoI and BamHI. The digested

DNA was ligated with a similarly digested bacterial pET 11d plasmid. The insert containing plasmid was used to transform *E. coli* DH5 α. Plasmid containing colonies were selected by growth on LB agar containing ampicillin. Plasmid DNA was isolated and sequenced. The sequence was as predicted as that of HcTnI followed directly by that of the published sequence for carp parvalbumin.

Plasmid DNA was used to transform *E. coli* BL21 (DE3). Cultures were grown in enriched media to mid log phase and induced with 1 mM Tris, pH 8.0, 0.1M NaCl, 0.1 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin. The sonicate was centrifuged at 48,000 xg and the supernatant was loaded on a S-Sepharose column equilibrated with the same buffer. The protein bound to the column and was eluted with a salt gradient.

Fractions containing HcTnI-Pv were pooled and dialyzed against 4 liters 4M urea, 1M NaCl, 50 mM Tris, pH 7.5, 1 mM DTT, 2 mM $CaCl_2$, 4 liters of the above buffer with 2M urea and 2 x 4 liters of above buffer without urea. The dialyzed pool was loaded on the TnC affinity column equilibrated with the above buffer without urea. The protein bound to the column and was eluted with a 100:100 ml gradient 0-6M urea, 0-3 mM EDTA. The protein was judged to be pure by SDS-PAGE.

The DNA sequence of this modification, HcTnI-Pv, is shown in SEQ ID NO: 12 and the deduced amino acid sequence is shown in SEQ ID NO: 13. Nucleotide sequence positions #631-633 of the DNA sequence of HcTnI represents the translational stop codon. This codon terminates translation of the messenger into protein and must not appear at this position in the fusion proteins or translation will be terminated before the full length protein is produced.

EXAMPLE 5: HcTnI MODIFIED WITH A HISTIDINE LEUCINE PEPTIDE

Recombinant HcTnI was modified to add an alternating 3 histidine 3 leucine tag to the C-terminal end of the molecule (HcTnI-$[HL]_3$). This modification was made to change the isoelectric point of the protein, thereby improving the solubility and stability of the resulting molecule.

The cDNA encoding this protein was synthesized by PCR using the described recombinant HcTnI [SEQ ID NO: 1] as the template, a 5' primer encoding part of the plasmid upstream of the insert, and as a 3' primer a synthesized oligonucleotide: 5'GTGGATCCTCAGAGATGGAGATGGAGATGGCTCT CAAACTTTTTCTTGCGG 3' [SEQ ID NO: 14]. The sequence of this 3' oligo reading from its 3' end contains a sequence complementary to the nucleotides 630-609 in the HcTnI sequence of SEQ ID NO: 1 (underlined), followed by codons coding for 3 sets of alternating histidines and leucines, a translational stop codon and a BamHI restriction site.

The PCR product was ligated to a bacterial pET 11d plasmid as described in detail for the other HcTnI modifications sequenced and expressed. The DNA sequence of this modification, HcTnI-$[HL]_3$, is shown in SEQ ID NO: 15 and the deduced amino acid sequence is shown in SEQ ID NO: 16.

EXAMPLE 6: DIALYSIS OF HcTnI's IN LOW IONIC STRENGTH SOLUTIONS FOLLOWED BY LYOPHILIZATION AND RECONSTITUTION

Aliquots of recombinant human cardiac HcTnI, HcTnI-$K_6$-$H_5$-D, HcTnI-Pv and HcTnI-HcTnC were prepared and purified as described in Examples 1-4 above. Each protein was dialyzed over several days against 4 times 4 liters of 1

mM HCl. Recombinant rabbit skeletal troponin T (rskTnT) was also expressed and purified as described in Potter, cited above, and then dialyzed as described for the TnI's above.

Each protein suspension was then aliquoted and lyophilized. Immediately upon completion of lyophilization, a set of aliquots of each protein was resuspended in a volume of distilled water equal to the aliquot volume before lyophilization. Additional aliquots of each lyophilized protein were resuspended after storage at -20°C for one week and one month. Samples of the protein at each stage of the process were kept at -80°C for later analysis.

The protein concentrations and reactivity with a monoclonal antibody before and after dialysis, lyophilization and reconstitution were tested as described below. Approximate concentration of each of the proteins before dialysis, after dialysis and after each reconstitution were determined as follows. The concentration of all protein suspensions was determined by densitometry on SDS PAGE (UVP Imagestore and Sigma Gel densitometry software, Jandel Scientific, San Rafael, CA). In order to estimate the concentration of the modified TnI's, the recombinant (wild-type) HcTnI [SEQ ID NO: 1] was used as a standard. This HcTnI is unmodified as cloned from human cardiac tissue [Armour *et al*, cited above]. This wild type TnI preparation was accepted to be at a concentration of 0.6 mg/mL as determined by BCA assay (Pierce, Rockford, IL), Coomassie assay and extinction. Conventional protein assays are commercially available from BioRAd, Hercules, CA, or otherwise are well known in the art (extinction coefficients).

The wild type, recombinant HcTnI was loaded on to an SDS gel in decreasing volumes in order to establish a calibration curve. This curve was then used to determine the concentration of the modified TnI's that were loaded on to the same gel. The values obtained from this experiment were used as the starting values for all the TnI's. These values were then used to set up calibration curves for the individual proteins to compare the concentrations after each step of dialysis, lyophilization and reconstitution.

*A. DIALYSIS*

The results of the protein determinations performed at each stage in the dialysis process are summarized in Table I below. Table I summarizes the quantitation of the proteins before and after dialysis in 1mM HCl. "Total Protein Before Dialysis" refers to the amount of each protein obtained after purification by column chromatography. Each of the proteins were in solutions containing 6M urea and a salt concentration ranging from 0.3 M to 1M at this stage. "Total Protein After Dialysis" refers to the amount of protein remaining after dialysis in 1mM HCl.

TABLE I

| PROTEIN | TOTAL PROTEIN BEFORE DIALYSIS | TOTAL PROTEIN AFTER DIALYSIS |
|---|---|---|
| WILD TYPE | 5.8 mg | 5.64 mg |
| HcTnI-$K_6$-$H_5$-D | 5.4 mg | 5.28 mg |
| HcTnI-Pv | 0.78 mg | 0.69 mg |
| HcTnI-HcTnC | 1.6 mg | 1.5 mg |

The results of Table I demonstrate that the proteins are able to be dialyzed into a low ionic strength

solution, not previously considered possible due to the insolubility of troponin I in low ionic strength solutions. In each case the volume of the aliquot increased, however, no loss of total protein was detected. Dialysis thus does not destroy or lose the protein.

*B. CONCENTRATION AFTER DIALYSIS, LYOPHILIZATION AND RECONSTITUTION*

The results of the protein determinations performed after dialysis, after lyophilization and after reconstitution are summarized in Table IA below. In Table IA aliquots of each protein dialyzed as above, were lyophilized and reconstituted in a volume of water equal to the original volume. Reconstitution occurred either immediately following lyophilization (concentration after reconstitution) or after storage for one week at -20°C (concentration after storage and reconstitution).

TABLE IA

| PROTEIN | CONCENTRATION AFTER DIALYSIS | CONCENTRATION AFTER RECONSTITUTION | CONCENTRATION AFTER STORAGE AND RECONSTITUTION |
|---|---|---|---|
| WILD TYPE | 0.47 mg/mL | 0.49mg/mL | 0.52 mg/mL |
| HcTnI-$K_6$-$H_5$-D | 0.55 mg/mL | 0.53 mg/mL | 0.49 mg/mL |
| HcTnI-Pv | 0.053 mg/mL | 0.054 mg/mL | 0.054 mg/mL |
| HcTnI-HcTnC | 0.3 mg/mL | not available | 0.25 mg/mL |

The results of Table IA demonstrate that the dialyzed solutions of TnI and modified TnI were able to be lyophilized and resuspended without loss of protein from precipitation or inability to resolubilize after lyophilization.

Protein samples were also stored for 1 month at -80°C and are described herein in Tables VI-VIII below.

EXAMPLE 7: IMMUNOREACTIVITY OF THE HcTnI PREPARATIONS

The proteins identified in Table I above were used to set up ELISA assays to determine the immunoreactivity of each protein after dialysis, after lyophilization and after reconstitution. In the ELISA plate assay procedure used herein the proteins (200 fmol, 100 fmol, and 50 fmol of each protein) were bound in quadruplicate to the wells on a plastic ELISA plate by passive absorption. A monoclonal antibody, 2A7-1E7 specific for HcTnI was reacted with the protein bound to the plate at a concentration of 5 pmole of antibody/well for one hour at room temperature. Examples of suitable anti-HcTnI antibodies are described in Larue *et al*, cited above.

After washing, the binding of the primary monoclonal antibody to the proteins was visualized by reacting the bound antibody with a secondary antibody, i.e., goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO). The amount of primary antibody bound is detected by addition of the chromogenic substrate 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt substrate (Sigma, St. Louis, MO) for the enzyme label on the secondary antibody. The amount of color produced is measured spectrophotometrically at 405 nm and is proportional to the amount of primary antibody bound.

Tables II through V below report the immunoreactivity results for each protein, at varying concentrations: (1) in the dialyzed solutions according to the invention; (2) after lyophilization of the dialyzed solutions and immediate reconstitution following lyophilization (first reconstitution); and (3) after the dialyzed solution is lyophilized and stored for one week at -20°C prior to

reconstitution (second reconstitution). In the tables below, the percents in the parenthesis are the percent of immunoreactivity in each preparation as compared to the reactivity after dialysis but before lyophilization (100% in all cases). Values listed are the mean of four duplicate wells. The optical density at a wavelength of 405 nanometers was measured. Due to the method of quantitation, the tables compare a particular protein to itself, after dialysis, and both before and after lyophilization. The tables do not compare results between proteins.

TABLE II
IMMUNOREACTIVITY OF WILD TYPE RECOMBINANT HcTnI

| WILD TYPE HcTnI | 200 fmol OD405 | 100 fmol OD405 | 50 fmol OD405 |
|---|---|---|---|
| AFTER DIALYSIS | 0.293 (100%) | 0.140 (100%) | 0.06 (100%) |
| FIRST RECONSTITUTION | 0.287 (98%) | 0.122 (87%) | 0.055 (92%) |
| SECOND RECONSTITUTION | 0.286 (98%) | 0.126 (90%) | 0.06 (100%) |

TABLE III
IMMUNOREACTIVITY OF HcTnI-$K_6$-$H_5$-D

| HcTnI-$K_6$-$H_5$-D | 200 fmol OD405 | 100 fmol OD405 | 50 fmol OD405 |
|---|---|---|---|
| AFTER DIALYSIS | 0.201 (100%) | 0.082 (100%) | 0.039 (100%) |
| FIRST RECONSTITUTION | 0.220 (109%) | 0.107 (130%) | 0.056 (144%) |
| SECOND RECONSTITUTION | 0.235 (117%) | 0.096 (117%) | 0.046 (118%) |

TABLE IV
IMMUNOREACTIVITY OF HcTnI-Pv

| HcTnI-Pv | 200 fmol OD405 | 100 fmol OD405 | 50 fmol OD405 |
|---|---|---|---|
| AFTER DIALYSIS | 1.0565 (100%) | 0.798 (100%) | 0.475 (100%) |
| FIRST RECONSTITUTION | 1.0325 (98%) | 0.844 (106%) | 0.473 (92%) |
| SECOND RECONSTITUTION | 1.005 (95%) | 0.698 (87%) | 0.374 (78%) |

TABLE V
IMMUNOREACTIVITY OF HcTnI-HcTnC

| HcTnI-Pv | 200 fmol OD405 | 100 fmol OD405 | 50 fmol OD405 |
|---|---|---|---|
| AFTER DIALYSIS | 0.682 (100%) | 0.223 (100%) | 0.081 (100%) |
| FIRST RECONSTITUTION | 0.583 (85%) | 0.167 (75%) | 0.055 (68%) |
| SECOND RECONSTITUTION | 0.666 (98%) | 0.259 (116%) | 0.096 (119%) |

The results presented in Tables II-V demonstrate that all of the proteins have comparable immunoreactivity after dialysis according to this invention, and after lyophilization and reconstitution to the same protein after dialysis but before lyophilization. The fairly low amounts of protein, i.e. 200-50 fmoles, were illustrated since if differences in reactivity were present, most likely, they would not be seen at higher concentration of protein. The HcTnI proteins and modified versions thereof are able to be dialyzed according to this invention, as well as lyophilized and resuspended without loss of immunoreactivity and with no alteration in antigenicity.

Therefore, stable standards and calibrators for use in a cardiac troponin I clinical immunoassays can be produced by preparing a dialyzed solution according to one

embodiment of this invention, or preparing a dialyzed, lyophilized composition, reconstitutable in water according to another embodiment of this invention.

Although only certain embodiments of the invention were tested, one of skill in the art can readily expect that other troponin proteins, modified proteins, fusion proteins and complexes described by this invention will also provide stable dialyzed and dialyzed and lyophilized standards for use in diagnostic assays.

EXAMPLE 8: STABILITY OF HcTnI PREPARATIONS OF THE PRIOR ART

This experiment demonstrates the stability of the HcTnI and modified HcTnI preparations as defined by the prior art, namely in 6M urea and high ionic strength solutions. Aliquots of the proteins were stored at either -80°C or 0°C in 6M urea and high ionic strength solutions. Results show that the proteins are stable at -80°C in these solutions. However, at temperatures of 0°C, the integrity of wild type HcTnI (Table IV) and, to a lesser degree, HcTnI-parvalbumin fusion protein (Table VII) is lost as demonstrated by the loss of immunoreactivity. HcTnI-$K_6H_5D$ appears resistent to breakdown under these conditions perhaps as a result of the carboxy terminus modification (Table VIII).

When compared to the results obtained with the same proteins treated according to the invention (Tables II-V), it is clear that the troponin proteins which are dialyzed and optionally lyophilized according to this invention illustrate significant improvement in stability over comparative prior art compositions.

Table VI illustrates the stability of wild type HcTnI stored at -80°C in the presence of 6M urea and high salt and HcTnI stored at 0°C in the presence of 6M urea and high salt.

TABLE VI

| Wild type | 500 fmol OD405 | 250 fmol OD405 | 125 fmol OD405 |
|---|---|---|---|
| stored at -80C | 0.483 | 0.193 | 0.074 |
| stored at 0C | 0.255 | 0.102 | 0.035 |

Table VII illustrates the stability of HcTnI-$K_6H_5D$ stored at -80°C in the presence of 6M urea and high salt and HcTnI-$K_6H_5D$ stored at 0°C in the presence of 6M urea and high salt.

TABLE VII

| HcTnI-KH | 55 fmol OD405 | 250 fmol OD405 | 125 fmol OD405 |
|---|---|---|---|
| stored at -80C | 0.240 | 0.094 | 0.034 |
| stored at 0C | 0.297 | 0.103 | 0.043 |

Table 8 illustrates the stability of HcTnI-Pv stored at -80°C in the presence of 6M urea and high salt and HcTnI-Pv stored at 0°C in the presence of 6M urea and high salt.

TABLE VIII

| HcTnI-Pv | 500 fmol OD405 | 250 fmol OD405 | 125 fmol OD405 |
|---|---|---|---|
| stored at -80C | 0.933 | 0.762 | 0.514 |
| stored at 0C | 0.955 | 0.630 | 0.319 |

The results shown in Tables VI-VIII demonstrate that immunoreactivity of wild type and HcTnI-Pv fusion protein decreases with storage at 0°C, even in the presence of 6M urea, 1M NaCl, 3 mM EDTA and 0.1 mM DTT, indicating the breakdown of these proteins over time. As HcTnI has been reported to be extremely susceptable to proteolysis (Hayden *et al*), this result was to be expected. This susceptability to proteolysis has been a limitation to the use of HcTnI as a standard and/or calibrator in clinical assays. Storage at -80°C in the presence of urea and high salt has previously been necessary in order to avoid breakdown of HcTnI. These results demonstrate that lyophilization and storage at -20°C in the absence of urea and high salt is comparable to storage at -80°C in the presence of urea and high salt. Also, these results indicate that the addition of the polylysine-histidine tail to the HcTnI-KH modification of wild type TnI may have stabilized the HcTnI molecule enough to be stored at 0°C in urea without the loss of immunoreactivity. However, this molecule can also be stored under the conditions of the present invention just as well.

EXAMPLE 9: ADVANTAGES OF HcTnI-Pv

The HcTnI-Pv molecule, which was not dialyzed and/or lyophilized according to one embodiment of this invention, was shown to have an additional advantageous characteristic in that it is not susceptible to the formation of interchain disulfide bonds as the other three HcTnI molecules. Fig. 1 shows the proteins on SDS-PAGE in the absence of the reducing agent, ß- mercaptoethanol. All of the HcTnI's show bands at the position of dimers formed from these molecules with the exception of HcTnI-Pv. This is a significant characteristic, as the oxidation of rabbit skeletal TnI has been shown to occur in dimeric complexes with TnC or TnT as well in whole troponin [Kluwe, L *et al*, **1993**, FEBS Lett., 323:83-88]. It may be inferred from this observation that oxidation of HcTnI may occur in complexes as well.

EXAMPLE 10: RECOMBINANT HUMAN CARDIAC TROPONIN T

In addition to the processes for stabilizing and solubilizing HcTnI for use as a calibrator/control standard in assays detecting HcTnI in the circulation of patients suspected of AMI, the same processes of the invention are applicable to other human troponin compounds including cardiac troponin T (HcTnT).

At present, there are clinical assays that detect HcTnT in the circulation of suspected AMI patients. The calibrator/standard utilized in these assays is bovine cardiac troponin T [Wu *et al.*, cited above]. An improvement over the prior art is the provision by this invention of HcTnT as the calibrator/control standard. The use of HcTnT in a

clinical assay format is presently problematic for the same reasons as described for human cardiac troponin I, i.e., inadequate amounts of tissue for isolation of the native molecule; difficulties in purification due to the instability and insolubility of both the native and recombinant molecules.

There are four isoforms of HcTnT; the most prevalent isoform in the adult heart has been reported to be an isoform containing a variable 15 nucleotide sequence. This isoform is called T3 [Anderson, P., *et al*, cited above]. Recombinant human cardiac troponin T (HcTnT), isoform 3, was cloned by reverse transcriptase PCR as described for HcTnI above. HcTnT was made by extraction of total RNA from adult human heart [Chomcynski and Sacci, cited above]. The RNA was transcribed into a cDNA copies using reverse transcriptase, cDNA Cycle Kit for RT-PCR (Invitrogen, San Diego, CA).

A 3' oligo complementary to the 3' end of the coding region of the known human troponin T gene [Anderson, P. *et al*, cited above] was synthesized for use as the annealing primer for synthesis of the first strand of cDNA. The oligo included the translational stop codon and a sequence representing a BamHI restriction site. The first strand of cDNA generated by reverse transcriptase was then used as a template for PCR amplification. The PCR reaction was primed using the previously described 3' oligonucleotide and a 5' oligonucleotide synthesized to correspond to the 5' end of the coding region for cTnT. The 5' oligo represents the first 20 nucleotides of HcTnT with a modification to include an NcoI site (CCATGG). This modification results in

a change in the second amino acid from serine to alanine. Each primer contained a restriction site at its 5' end to facilitate subsequent subcloning.

The PCR cycles were as follows: 2 minutes at 94° C, 30 cycles of 30 seconds at 95° C, 2 minutes at 50°C, 2 minutes at 72°C with the last cycle ending in 10 minutes at 72°C. The PCR product was isolated on a low melt agarose gel, cut out and cleaned with gene clean (BIO 101, Vista, CA). The product and a prokaryotic plasmid vector containing a multiple cloning site were digested with NeoI and Bam HI. The two DNA strands were ligated using T4 DNA ligase (Boerhinger Mannheim, Indianapolis, IN) and *E. coli* DH5α was transformed with the insert containing plasmid.

Colonies of DH5 α that contained the plasmid were selected by growth on LB agar containing ampicillin. The plasmid contains the sequence coding for ß lactamase production therefore conferring resistance to ampicillin to the transformed bacteria only. The plasmid DNA was isolated from the host bacteria by standard alkaline lysis method and sequenced (Sequenase, United States Biochemical, Cleveland, OH). The sequence of the troponin T ($HcTnT_3$) cDNA was found to be identical to the published sequence for the predominant adult isoform of this molecule, referred to as isoform T [Anderson *et al*, cited above].

The expression host, *E. coli* BL21(DE3) pLysS was then transformed with the vector. The culture was grown in enriched media (Yeast extract, tryptone and M9 minimal slats, Sigma, St. Louis, MI) in the

presence of 200 µg/ml ampicillin (plasmid resistance marker) to mid log phase and induced by making the culture 1 mM in TPTG (Boehringer Mannheim, Indianapolis, IN). Recombinant human cardiac troponin T was isolated from the bacterial culture by sonication of the bacterial pellet in 6M urea, 10 mM Sodium citrate, pH 7.0, 2 mM EDTA, 1mM DTT. The sonicate was centrifuged at 48,000 xg. The supernatant was then adjusted to pH 5.0 and centrifuged again. The supernatant from this spin was loaded on an S-Sepharose column equilibrated in the same buffer. The column was eluted with a 600:600 ml, 0-0.6M NaCl gradient. The fractions containing HcTnT were pooled and dialyzed against 4 X 4 liters of 6M urea, 20 mM Tris, pH 7.8, 1mM EDTA and 0.3 mM DTT and loaded on to a Q-Sepharose column. The column was equilibrated in the same buffer before the protein was loaded. After the protein was bound the column was washed and eluted with a 500:500ml, 0-1MNaCl gradient Human cTnT was judged to be pure by SDS-PAGE.

The sequence of HcTnT isoform T3 is shown in SEQ ID NO: 17; and the deduced amino acid sequence is shown in SEQ ID NO: 18.

EXAMPLE 11: STABILITY OF HcTnT

Recombinant HcTnT dialyzed according to the present invention into a stable solution, or dialyzed and lyophilized according to the present invention, provides readily available, stable and soluble preparations of HcTnT for use in diagnostic assays.

Aliquots of recombinant human cardiac TnT, purified as defined above were dialyzed over several

days against 4 liters of 1 mM HCl changing the solution four times. The aliquots of the dialyzed proteins were either left in the liquid state or lyophilized. Liquid and lyophilized samples were stored for at least 30 days at either ambient temperature, 4°C or -20°C. After the 30 day storage period, the lyophilized samples were reconstituted with distilled water and all of the samples were checked for integrity on SDS-PAGE and immunoreactivity utilizing Boehringer Mannheim's ELISA cardiac TnT assay.

The results of SDS-PAGE gel are shown in Fig. 5. The liquid or lyophilized reconstituted samples, stored for at least 30 days at ambient temperature, 4°C or -20° C showed minimal loss of protein as judged by SDS-PAGE.

EXAMPLE 12: IMMUNOREACTIVITY OF rHcTnT

The immunological reactivity of the recombinant human cardiac troponin T (cTnT) of Example 11, which was both dialyzed and lyophilized as described herein, was evaluated in a microplate ELISA using Enzymun-Test® Troponin-T (Boehringer Mannheim, Catalog No. 1 556 428) reagents prepared as described in the product insert and run on streptavidin plates (MicroCoat Beshichtungstechnik, Catalog No. 148 7051). The cTnT was diluted 16,000-fold in adenin-citrate dextrose plasma. Forty microliters of the standards, controls and the diluted samples were placed in the appropriate wells and the binding incubation was initiated with 200µL of the Anti-Tn-T-POD conjugate. The plate was incubated for one hour

at ambient temperature and washed with 5 times 200µL of the Washing Solution. The assay response was detected with 200µL of the Substrate-chromogen solution and the absorbance was read at 405nm at 15 minutes. Sample concentrations were interpolated from the standard curve and corrected for dilution.

The results of the ELISA assay are shown in Table IX, which reports the conditions under which the HcTnT were assayed. Where the indication of "lyophilized" in the table means dialyzed as required by the invention and lyophilized.

TABLE IX

| DAY | STD SET | CONDITION | ASSAY RESULTS uG/mL TnT |
|---|---|---|---|
| 0 | 1 | ORIGINAL | 28.91 |
| 36 | 1 | RT liquid | 74.8 |
| 36 | 1 | 4°C liquid | 55.58 |
| 36 | 1 | -20°C liquid | 50.24 |
| 36 | 1 | RT lyophilized | 79.99 |
| 36 | 1 | 4°C lyophilized | 58.35 |
| 36 | 1 | -20°C lyophilized | 45.53 |
| 36 | 2 | RT liquid | 76.28 |
| 36 | 2 | 4°C liquid | 57.89 |
| 36 | 2 | -20°C liquid | 52.58 |
| 36 | 2 | RT lyophilized | 81.04 |
| 36 | 2 | 4°C lyophilized | 60.61 |
| 36 | 2 | -20°C lyophilized | 47.83 |

The results indicate that, under all conditions, the immunoreactivity of HcTnT, was maintained.

EXAMPLE 13: TROPONIN COMPLEXES

Complexes of recombinant human troponins are produced by expression of the proteins in bacterial expression hosts. Within the troponin complex (i.e., TnC-TnI-TnT in equimolar ratio) as it appears naturally in muscle, TnI and TnT are soluble in low ionic strength solutions. These molecules are also more stable with the complex than as isolated proteins [Larue *et al*, cited above; and Kluwe *et al*, cited above]. The production of a dimeric or trimeric complex using native human cardiac TnC, TnI and/or TnT has been described *in vitro* [Larue *et al*, cited above]. The construction of a vector expressing all three components of chicken skeletal troponin as a whole complex has also been described [Malnic, B. *et al*, 1994, Eur. J. Biochem., 224:49-54].

The present invention provides recombinant heterodimeric complexes of troponins as well as a recombinant heterotrimeric complex. These complexes are stable, soluble molecules that can be used as calibrator/control standard in clinical assays detecting HcTnI or HcTnT. These complexes may be prepared into stable dialyzed solutions according to one embodiment of the method of this invention. Alternatively, these complexes may be prepared into stable, dialyzed and lyophilized compositions according to another embodiment of the method of this invention.

The complexes are a further improvement over the current available troponin standards, as it is not known how the troponins, HcTnI and HcTnT are released into the circulation from the damaged heart tissue. It is possible and even probable that the proteins are released as a mixed population of individual components, whole and partial troponin complexes. For assay development and quality control to be assured, the selected method of detection employed by the selected assay will detect TnI or TnT when it is part of a complex as well as an isolated protein by use of the complexes of this invention.

*A. Heterodimeric TnI/TnC*

A bacterial plasmid has been constructed that contains the coding sequences for both HcTnI and HcTnC in tandem on the same plasmid. The plasmid vector, pET 11d described above, containing the coding sequence for HcTnC [SEQ ID NO:19] was digested with restriction enzymes Bgl II and Bam HI (Gibco, Gaithersburg, MD). The resulting DNA fragment consisted of the promoter region of the plasmid followed by the coding sequence for HcTnC. This fragment was isolated on an agarose gel, cut out and cleaned with gene clean (BIO 101, Vista, CA). A second pET vector, pET 3d, that contained the coding sequence for HcTnI was digested with Bam HI. This linearized plasmid was also cleaned as above and a ligation reaction was set up to join the promoter/TnC fragment with the linearized plasmid using T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN).

The enzymes Bam HI and Bgl II produce cuts that have complimentary overhangs, so that DNA digested with Bam HI can ligate to DNA digested with

Bgl II and vice versa. It was expected that a certain proportion of the ligated plasmids would contain the promoter/HcTnC sequence in the proper orientation for expression.

*E. coli* DH5 was transformed with the ligation reaction and transformed bacteria were selected by growth on LB agar plates containing ampicillin. Small plasmid preps were made from colonies growing on the agar plate and digested with Eco RV (Gibco, Gaithersburg, MD). This enzyme produced fragment lengths that indicate the presence and the orientation of the HcTnC/promoter DNA into the plasmid.

A plasmid prep shown to contain the desired restriction fragment lengths was then used to transform the expression host *E. coli* BL-21 (DE3) pLysS. Bacteria containing the plasmid were again selected on LB agar plates containing ampicillin. Cultures of colonies selected from this plate were then grown in enriched media containing ampicillin as described in the previous examples and checked for expression of the proteins by lysis of the centrifuged bacterial pellet in SDS-PAGE sample buffer and electrophoresis on SDS-PAGE gel.

All cultures tested demonstrated expression of both HcTnC and HcTnI (see Fig. 2). As illustrated in Fig. 2, the band corresponding to the position of HcTnI was not as heavy as that corresponding to HcTnC as is the case when these proteins are expressed separately. In order to confirm that the band corresponding to HcTnI was actually HcTnI and not a bacterial protein of the same molecular weight, a Western blot using a monoclonal antibody specific for

HcTnI was performed. The Western blot confirmed that the band was HcTnI. These two proteins, expressed in the same host, form a complex within the bacteria.

Large cultures of the bacteria containing the TnI/TnC construct were grown overnight. The bacteria were centrifuged and the pellets resuspended in 6M urea, 50 mM Tris pH8.0, 1 mM $CaCl_2$, 2 mM ß-mercaptoethanol. The resuspended bacteria were then sonicated for a total of 10 minutes and centrifuged at 48,000 x g for 20 minutes. The supernatant was dialyzed against 50 mM Tris, pH 8.0, 2 mM $CaCl_2$, 1 mM BME and partially purified on a DEAE Sepharose column equilibrated with the same buffer. The column was washed and eluted with a 500:500 mL gradient of 0-0.5M NaCl. TnI and TnC co-eluted in fractions 24 through 45 (Fig. 3), demonstrating the formation of a complex of these two proteins. Fig. 4 demonstrates the presence of HcTnI in fractions 24-45 by Western blot.

*B. Heterotrimeric HcTnT/HcTnI/HcTnC*

The HcTnT, as cloned, is expressed, purified and stabilized as described in the previous examples for the HcTnI proteins. The cDNA is also used as part of another construct expressing HcTnC, I and T together on one plasmid as described above for HcTnC and HcTnI. These molecules form the complete troponin complex as expressed by the bacteria.

After the TnI/TnC construct was made, the cDNA encoding HcTnT [SEQ ID NO:17] was introduced into the plasmid following the TnC insert. The plasmid containing TnI/TnC was cut with BamHI. A BglII/BamHI fragment containing the coding sequence for HcTnT was ligated with the linearized plasmid.

The ligation reaction was used to transform DH5 host bacteria and selected as previously described. Plasmids containing the proper size restriction fragments were selected and used to transform the expression host BL-21 (DE3) and BL-21 (DE3) pLysS. These bacteria were grown overnight and pelleted. The pellets were resuspended in SDS-PAGE buffer and electrophoresed. The proteins were transferred to nitrocellulose for detection by Western Blot. A single blot using antibodies against HcTnI, HcTnT and HcTnC demonstrated the expression of all three subunits from a single plasmid (see Fig. 6, lanes 1-3).

These multimeric complexes may be dialyzed into stable solutions and optionally lyophilized as described above. Thus, they are similarly useful as calibrators or controls for diagnostic assays.

EXAMPLE 14: STABILITY OF COMPLEXES

The stability of the troponin complex was evaluated by assembling the subunits *in vitro*. The subunits were mixed together in equimolar ratio. The HcTnI and HcTnT subunits were in 1 mM HCl and the HcTnC was in 50 mM Tris pH8.0, 1 mM $CaCl_2$. These subunits were then dialyzed against either HCl or 5mM ammonium bicarbonate, four changes of 1 liter each over a period of two days. Since no precipitate was observed in any of the dialyzed samples, it was concluded that the subunits had complexed. HcTnC precipitates at low pH and HcTnI and HcTnT are insoluble, as isolated subunits, in 5 mM ammonium bicarbonate. After dialysis, an aliquot was kept as a liquid, while the remaining volume was lyophilized.

An SDS-PAGE gel was run on these samples after dialysis was complete (see Fig. 7). As can be seen, all of the subunits are present in the correct stoichiometric ratio. The samples were then stored at 4°C if liquid or at ambient temperature if lyophilized for a period of two weeks. The lyophilized samples were then reconstituted in the same volume of distilled water and are shown in Fig. 8, lanes 1-4. As can be seen from the Fig. 8, all of the complexes are the same as after dialysis (Fig. 7). The liquid samples stored in 1 mM HCl were very stable and showed no degradation (Fig. 8, lane 8 - TnI/TnC; and lane 10 -TnT/TnI/TnC. However, the ammonium bicarbonate liquid samples were very unstable and TnT and TnI in these samples was completely degraded (Fig. 8, lanes 7 and 9).

All documents and publications referred to above and the contents of the sequence listing are incorporated by reference herein. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. For example, although full-length cardiac troponin I, C or T was the protein tested and reported in the Examples above, it is expected that functional fragments of the full-length proteins, or other carboxy-terminal modified versions of the proteins may be treated by this method and also retain stability for long periods under typical conditions of storage, e.g., elevated temperatures. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Miami

(ii) TITLE OF INVENTION: Stabilized Preparations of Human Troponins and Modifications Thereof, Diagnostic Assay Methods and Assay Kits

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Coulter International Corp.
(B) STREET: Mail Code 32-A02, P.O. Box 169015
(C) CITY: Miami
(D) STATE: Florida
(E) COUNTRY: USA
(F) ZIP: 33116-9015

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/015,772
(B) FILING DATE: 16-APR-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: 11-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kurz, Warren W.
(B) REGISTRATION NUMBER: 24,418
(C) REFERENCE/DOCKET NUMBER: COU1PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 305-380-2038
(B) TELEFAX: 305-380-4566

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 633 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..630

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GCG GAT GGG AGC AGC GAT GCG GCT AGG GAA CCT CGC CCT GCA CCA        48
Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

GCC CCA ATC AGA CGC CGC TCC TCC AAC TAC CGC GCT TAT GCC ACG GAG        96
Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG       144
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

CTG AAG ACT CTG CTG CTG CAG ATT GCA AAG CAA GAG CTG GAG CGA GAG       192
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

GCG GAG GAG CGG CGC GGA GAG AAG GGG CGC GCT CTG AGC ACC CGC TGC       240
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG       288
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC       336
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110

GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG       384
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG       432
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140

CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG       480
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160

GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG       528
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

AAG AAG GAG GAC ACC GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGG       576
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT       624
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
```

195 200 205

GAG AGC TGA 633
Glu Ser
210

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 210 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
1 5 10 15

Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
20 25 30

Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
35 40 45

Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
50 55 60

Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65 70 75 80

Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
85 90 95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
100 105 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
115 120 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
130 135 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145 150 155 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
165 170 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
180 185 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
195 200 205

Glu Ser
210

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGGATCCTC AGTGATGGTG ATGGTGATGT TCTTTTTCT TTTTCTTGCT CTCAAACTTT 60

TTCTTGCGG 69

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 669 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..666

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG GCG GAT GGG AGC AGC GAT GCG GCT AGG GAA CCT CGC CCT GCA CCA      48
Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

GCC CCA ATC AGA CGC CGC TCC TCC AAC TAC CGC GCT TAT GCC ACG GAG      96
Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG     144
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

CTG AAG ACT CTG CTG CTG CAG ATT GCA AAG CAA GAG CTG GAG CGA GAG     192
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

GCG GAG GAG CGG CGC GGA GAG AAG GGG CGC GCT CTG AGC ACC CGC TGC     240
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG     288
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
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```
                    85                  90                  95

TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC      336
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110

GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG      384
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG      432
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140

CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG      480
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160

GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG      528
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

AAG AAG GAG GAC ACC GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGG      576
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT      624
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
        195                 200                 205

GAG AGC AAG AAA AAG AAA AAG AAA CAT CAC CAT CAC CAT GAC               666
Glu Ser Lys Lys Lys Lys Lys Lys His His His His His Asp
    210                 215                 220

TGA                                                                  669
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60
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Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65 70 75 80

Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
85 90 95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
100 105 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
115 120 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
130 135 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145 150 155 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
165 170 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
180 185 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
195 200 205

Glu Ser Lys Lys Lys Lys Lys Lys His His His His His Asp
210 215 220

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCAGCCTTG TAGATGTCAT CCATGCTCTC AAACTTTTTC TTGCGGCCCT C 51

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGGCCGCAA GAAAAAGTTT GAGAGCATGG ATGACATCTA CAAGGCTGGC TGCGGTAG 58

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1116

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GTG GAT GGG AGC AGC GAT GCG GCT AGG GAA CCT CGC CCT GCA CCA      48
Met Val Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

GCC CCA ATC AGA CGC CGC TCC TCC AAC TAC CGC GCT TAT GCC ACT GAG      96
Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG     144
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

CTG AAG ACT CTG CTG CTG CAG ATT GCA AAG CAA GAG CTG GAG CGA GAG     192
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

GCG GAG GAG CGG CGC GGA GAG AAG GGG CGC GCT CTG AGC ACC CGC TGC     240
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG     288
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC     336
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110

GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG     384
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG     432
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140
```

```
CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG          480
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160

GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG          528
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

AAG AAG GAG GAC ACC GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGC          576
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT          624
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
        195                 200                 205

GAG AGC ATG GAT GAC ATC TAC AAG GCT GCG GTA GAG CAG CTG ACA GAA          672
Glu Ser Met Asp Asp Ile Tyr Lys Ala Ala Val Glu Gln Leu Thr Glu
    210                 215                 220

GAG CAG AAA AAT GAG TTC AAG GCA GCC TTC GAC ATC TTC GTG CTG GGC          720
Glu Gln Lys Asn Glu Phe Lys Ala Ala Phe Asp Ile Phe Val Leu Gly
225                 230                 235                 240

GCT GAG GAT GGC TGC ATC AGC ACC AAG GAG CTG GGC AAG GTG ATG AGG          768
Ala Glu Asp Gly Cys Ile Ser Thr Lys Glu Leu Gly Lys Val Met Arg
                245                 250                 255

ATG CTG GGC CAG AAC CCC ACC CCT GAG GAG CTG CAG GAG ATG ATC GAT          816
Met Leu Gly Gln Asn Pro Thr Pro Glu Glu Leu Gln Glu Met Ile Asp
            260                 265                 270

GAG GTG GAC GAG GAC GGC AGC GGC ACG GTG GAC TTT GAT GAG TTC CTG          864
Glu Val Asp Glu Asp Gly Ser Gly Thr Val Asp Phe Asp Glu Phe Leu
        275                 280                 285

GTC ATG ATG GTT CGG TGC ATG AAG GAC GAC AGC AAA GGG AAA TCT GAG          912
Val Met Met Val Arg Cys Met Lys Asp Asp Ser Lys Gly Lys Ser Glu
    290                 295                 300

GAG GAG CTG TCT GAC CTC TTC CGC ATG TTT GAC AAA AAT GCT GAT GGC          960
Glu Glu Leu Ser Asp Leu Phe Arg Met Phe Asp Lys Asn Ala Asp Gly
305                 310                 315                 320

TAC ATC GAC CTG GAT GAG CTG AAG ATA ATG CTG CAG GCT ACA GGC GAG         1008
Tyr Ile Asp Leu Asp Glu Leu Lys Ile Met Leu Gln Ala Thr Gly Glu
                325                 330                 335

ACC ATC ACG GAG GAC GAC GAC ATC GAG GAG CTC ATG AAG GAC GGA GAC         1056
Thr Ile Thr Glu Asp Asp Asp Ile Glu Glu Leu Met Lys Asp Gly Asp
            340                 345                 350

AAG AAC AAC GAC GGC CGC ATC GAC TAT GAT GAG TTC CTG GAG TTC ATG         1104
Lys Asn Asn Asp Gly Arg Ile Asp Tyr Asp Glu Phe Leu Glu Phe Met
        355                 360                 365

AAG GGG GTC GAG TAA                                                     1119
Lys Gly Val Glu
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 372 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Val Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
        195                 200                 205

Glu Ser Met Asp Asp Ile Tyr Lys Ala Ala Val Glu Gln Leu Thr Glu
    210                 215                 220

Glu Gln Lys Asn Glu Phe Lys Ala Ala Phe Asp Ile Phe Val Leu Gly
225                 230                 235                 240
```

Ala Glu Asp Gly Cys Ile Ser Thr Lys Glu Leu Gly Lys Val Met Arg
245 250 255

Met Leu Gly Gln Asn Pro Thr Pro Glu Glu Leu Gln Glu Met Ile Asp
260 265 270

Glu Val Asp Glu Asp Gly Ser Gly Thr Val Asp Phe Asp Glu Phe Leu
275 280 285

Val Met Met Val Arg Cys Met Lys Asp Asp Ser Lys Gly Lys Ser Glu
290 295 300

Glu Glu Leu Ser Asp Leu Phe Arg Met Phe Asp Lys Asn Ala Asp Gly
305 310 315 320

Tyr Ile Asp Leu Asp Glu Leu Lys Ile Met Leu Gln Ala Thr Gly Glu
325 330 335

Thr Ile Thr Glu Asp Asp Asp Ile Glu Glu Leu Met Lys Asp Gly Asp
340 345 350

Lys Asn Asn Asp Gly Arg Ile Asp Tyr Asp Glu Phe Leu Glu Phe Met
355 360 365

Lys Gly Val Glu
370

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCGTCGTTC AGAACACCAG CGAAAGCCAT GCTCTCAAAC TTTTTCTTGC GGCCCTC 57

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGGGCCGCAA GAAAAAGTTT GAGAGCATGG CTTTCGCTGG TGTTCTGAAC GACGCTG 57

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 960 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..957

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG GTG GAT GGG AGC AGC GAT GCG GCT AGG GAA CCT CGC CCT GCA CCA      48
Met Val Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

GCC CCA ATC AGA CGC CGC TCC TCC AAC TAC CGC GCT TAT GCC ACG GAG      96
Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG     144
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

CTG AAG ACT CTG CTG CTG CAG ATT GCA AAG CAA GAG CTG GAG CGA GAG     192
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

GCG GAG GAG CGG CGC GGA GAG AAG GGG CGC GCT CTG AGC ACC CGC TGC     240
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG     288
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC     336
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110

GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG     384
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG     432
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140

CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG     480
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160
```

```
GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG          528
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

AAG AAG GAG GAC ACC GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGC          576
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT          624
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
        195                 200                 205

GAG AGC ATG GCT TTC GCT GGT GTT CTG AAC GAC GCT GAC ATC GCT GCT          672
Glu Ser Met Ala Phe Ala Gly Val Leu Asn Asp Ala Asp Ile Ala Ala
    210                 215                 220

GCT CTG GAA GCA TGC AAA GCC GCG GAC TCT TTC AAC CAT AAA GCA TTC          720
Ala Leu Glu Ala Cys Lys Ala Ala Asp Ser Phe Asn His Lys Ala Phe
225                 230                 235                 240

TTT GCT AAA GTT GGT CTG ACC TCT AAA TCT GCT GAC GAC GTT AAA AAA          768
Phe Ala Lys Val Gly Leu Thr Ser Lys Ser Ala Asp Asp Val Lys Lys
                245                 250                 255

GCT TTC GCT ATC ATC GAC CAG GAC AAA TCT GGT TTC ATC GAA GAA GAC          816
Ala Phe Ala Ile Ile Asp Gln Asp Lys Ser Gly Phe Ile Glu Glu Asp
            260                 265                 270

GAA CTG AAA CTG TTC CTG CAG AAC TTT AAA GCT GAC GCG CGC GCT CTG          864
Glu Leu Lys Leu Phe Leu Gln Asn Phe Lys Ala Asp Ala Arg Ala Leu
        275                 280                 285

ACC GAC GGT GAA ACC AAA ACC TTT CTG AAA GCT GGT GAC TCT GAC GGT          912
Thr Asp Gly Glu Thr Lys Thr Phe Leu Lys Ala Gly Asp Ser Asp Gly
    290                 295                 300

GAC GGT AAA ATC GGT GTT GAC GAA TTC ACC GCT CTG GTT AAA GCA              957
Asp Gly Lys Ile Gly Val Asp Glu Phe Thr Ala Leu Val Lys Ala
305                 310                 315

TGA                                                                     960
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 319 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Val Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
  1               5                  10                  15

Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
```

20 25 30

Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
35 40 45

Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
50 55 60

Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65 70 75 80

Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
85 90 95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
100 105 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
115 120 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
130 135 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145 150 155 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
165 170 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
180 185 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
195 200 205

Glu Ser Met Ala Phe Ala Gly Val Leu Asn Asp Ala Asp Ile Ala Ala
210 215 220

Ala Leu Glu Ala Cys Lys Ala Ala Asp Ser Phe Asn His Lys Ala Phe
225 230 235 240

Phe Ala Lys Val Gly Leu Thr Ser Lys Ser Ala Asp Asp Val Lys Lys
245 250 255

Ala Phe Ala Ile Ile Asp Gln Asp Lys Ser Gly Phe Ile Glu Glu Asp
260 265 270

Glu Leu Lys Leu Phe Leu Gln Asn Phe Lys Ala Asp Ala Arg Ala Leu
275 280 285

Thr Asp Gly Glu Thr Lys Thr Phe Leu Lys Ala Gly Asp Ser Asp Gly
290 295 300

Asp Gly Lys Ile Gly Val Asp Glu Phe Thr Ala Leu Val Lys Ala
305 310 315

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGGATCCTC AGAGATGGAG ATGGAGATGG CTCTCAAACT TTTTCTTGCG G 51

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 651 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..648

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG GCG GAT GGG AGC AGC GAT GCG GCT AGG GAA CCT CGC CCT GCA CCA      48
Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

GCC CCA ATC AGA CGC CGC TCC TCC AAC TAC CGC GCT TAT GCC ACG GAG      96
Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

CCG CAC GCC AAG AAA AAA TCT AAG ATC TCC GCC TCG AGA AAA TTG CAG     144
Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

CTG AAG ACT CTG CTG CTG CAG ATT GCA AAG CAA GAG CTG GAG CGA GAG     192
Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

GCG GAG GAG CGG CGC GGA GAG AAG GGG CGC GCT CTG AGC ACC CGC TGC     240
Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

CAG CCG CTG GAG TTG GCC GGG CTG GGC TTC GCG GAG CTG CAG GAC TTG     288
Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

TGC CGA CAG CTC CAC GCC CGT GTG GAC AAG GTG GAT GAA GAG AGA TAC     336
Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
            100                 105                 110
```

```
GAC ATA GAG GCA AAA GTC ACC AAG AAC ATC ACG GAG ATT GCA GAT CTG          384
Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
        115                 120                 125

ACT CAG AAG ATC TTT GAC CTT CGA GGC AAG TTT AAG CGG CCC ACC CTG          432
Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
    130                 135                 140

CGG AGA GTG AGG ATC TCT GCA GAT GCC ATG ATG CAG GCG CTG CTG GGG          480
Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145                 150                 155                 160

GCC CGG GCT AAG GAG TCC CTG GAC CTG CGG GCC CAC CTC AAG CAG GTG          528
Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
                165                 170                 175

AAG AAG GAG GAC ACC GAG AAG GAA AAC CGG GAG GTG GGA GAC TGG CGG          576
Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
            180                 185                 190

AAG AAC ATC GAT GCA CTG AGT GGA ATG GAG GGC CGC AAG AAA AAG TTT          624
Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
        195                 200                 205

GAG AGC CAT CTC CAT CTC CAT CTC TGA                                      651
Glu Ser His Leu His Leu His Leu
    210                 215
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 216 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Met Ala Asp Gly Ser Ser Asp Ala Ala Arg Glu Pro Arg Pro Ala Pro
 1               5                  10                  15

Ala Pro Ile Arg Arg Arg Ser Ser Asn Tyr Arg Ala Tyr Ala Thr Glu
            20                  25                  30

Pro His Ala Lys Lys Lys Ser Lys Ile Ser Ala Ser Arg Lys Leu Gln
        35                  40                  45

Leu Lys Thr Leu Leu Leu Gln Ile Ala Lys Gln Glu Leu Glu Arg Glu
    50                  55                  60

Ala Glu Glu Arg Arg Gly Glu Lys Gly Arg Ala Leu Ser Thr Arg Cys
65                  70                  75                  80

Gln Pro Leu Glu Leu Ala Gly Leu Gly Phe Ala Glu Leu Gln Asp Leu
                85                  90                  95

Cys Arg Gln Leu His Ala Arg Val Asp Lys Val Asp Glu Glu Arg Tyr
```

100 105 110

Asp Ile Glu Ala Lys Val Thr Lys Asn Ile Thr Glu Ile Ala Asp Leu
115 120 125

Thr Gln Lys Ile Phe Asp Leu Arg Gly Lys Phe Lys Arg Pro Thr Leu
130 135 140

Arg Arg Val Arg Ile Ser Ala Asp Ala Met Met Gln Ala Leu Leu Gly
145 150 155 160

Ala Arg Ala Lys Glu Ser Leu Asp Leu Arg Ala His Leu Lys Gln Val
165 170 175

Lys Lys Glu Asp Thr Glu Lys Glu Asn Arg Glu Val Gly Asp Trp Arg
180 185 190

Lys Asn Ile Asp Ala Leu Ser Gly Met Glu Gly Arg Lys Lys Lys Phe
195 200 205

Glu Ser His Leu His Leu His Leu
210 215

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 867 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..864

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG TCT GAC ATA GAA GAG GTG GTG GAA GAG TAC GAG GAG GAG GAG CAG 48
Met Ser Asp Ile Glu Glu Val Val Glu Glu Tyr Glu Glu Glu Glu Gln
1 5 10 15

GAA GAA GCA GCT GTT GAA GAG CAG GAG GAG GCA GCG GAA GAG GAT GCT 96
Glu Glu Ala Ala Val Glu Glu Gln Glu Glu Ala Ala Glu Glu Asp Ala
20 25 30

GAA GCA GAG GCT GAG ACC GAG GAG ACC AGG GCA GAA GAA GAT GAA GAA 144
Glu Ala Glu Ala Glu Thr Glu Glu Thr Arg Ala Glu Glu Asp Glu Glu
35 40 45

GAA GAG GAA GCA AAG GAG GCT GAA GAT GGC CCA ATG GAG GAG TCC AAA 192
Glu Glu Glu Ala Lys Glu Ala Glu Asp Gly Pro Met Glu Glu Ser Lys
50 55 60

CCA AAG CCC AGG TCG TTC ATG CCC AAC TTG GTG CCT CCC AAG ATC CCC 240
Pro Lys Pro Arg Ser Phe Met Pro Asn Leu Val Pro Pro Lys Ile Pro

```
65                  70                  75                  80

GAT GGA GAG AGA GTG GAC TTT GAT GAC ATC CAC CGG AAG CGC ATG GAG      288
Asp Gly Glu Arg Val Asp Phe Asp Asp Ile His Arg Lys Arg Met Glu
                85                  90                  95

AAG GAC CTG AAT GAG TTG CAG GCG CTG ATT GAG GCT CAC TTT GAG AAC      336
Lys Asp Leu Asn Glu Leu Gln Ala Leu Ile Glu Ala His Phe Glu Asn
            100                 105                 110

AGG AAG AAA GAG GAG GAG GAG CTC GTT TCT CTC AAA GAC AGG ATC GAG      384
Arg Lys Lys Glu Glu Glu Glu Leu Val Ser Leu Lys Asp Arg Ile Glu
        115                 120                 125

AGA CGT CGG GCA GAG CGG GCC GAG CAG CAG CGC ATC CGG AAT GAG CGG      432
Arg Arg Arg Ala Glu Arg Ala Glu Gln Gln Arg Ile Arg Asn Glu Arg
    130                 135                 140

GAG AAG GAG CGG CAG AAC CGC CTG GCT GAA GAG AGG GCT CGA CGA GAG      480
Glu Lys Glu Arg Gln Asn Arg Leu Ala Glu Glu Arg Ala Arg Arg Glu
145                 150                 155                 160

GAG GAG GAG AAC AGG AGG AAG GCT CAG GAT GAG GCC CGG AAG AAG AAG      528
Glu Glu Glu Asn Arg Arg Lys Ala Gln Asp Glu Ala Arg Lys Lys Lys
                165                 170                 175

GCT TTG TCC AAC ATG ATG CAT TTT GGG GGT TAC ATC CAG AAG CAG GCC      576
Ala Leu Ser Asn Met Met His Phe Gly Gly Tyr Ile Gln Lys Gln Ala
            180                 185                 190

CAG ACA GAG CGG AAA AGT GGG AAG AGG CAG ACT GAG CGG GAA AAG AAG      624
Gln Thr Glu Arg Lys Ser Gly Lys Arg Gln Thr Glu Arg Glu Lys Lys
        195                 200                 205

AAG AAG ATT CTG GCT GAG AGG AGG AAG GTG CTG GCC ATT GAC CAC CTG      672
Lys Lys Ile Leu Ala Glu Arg Arg Lys Val Leu Ala Ile Asp His Leu
    210                 215                 220

AAT GAA GAT CAG CTG AGG GAG AAG GCC AAG GAG CTG TGG CAG AGC ATC      720
Asn Glu Asp Gln Leu Arg Glu Lys Ala Lys Glu Leu Trp Gln Ser Ile
225                 230                 235                 240

TAT AAC TTG GAG GCA GAG AAG TTC GAC CTG CAG GAG AAG TTC AAG CAG      768
Tyr Asn Leu Glu Ala Glu Lys Phe Asp Leu Gln Glu Lys Phe Lys Gln
                245                 250                 255

CAG AAA TAT GAG ATC AAT GTT CTC CGA AAC AGG ATC AAC GAT AAC CAG      816
Gln Lys Tyr Glu Ile Asn Val Leu Arg Asn Arg Ile Asn Asp Asn Gln
            260                 265                 270

AAA GTC TCC AAG ACC CGC GGG AAG GCT AAA GTC ACC GGG CGC TGG AAA      864
Lys Val Ser Lys Thr Arg Gly Lys Ala Lys Val Thr Gly Arg Trp Lys
        275                 280                 285

TAG                                                                  867
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 288 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Ser Asp Ile Glu Glu Val Val Glu Glu Tyr Glu Glu Glu Glu Gln
 1               5                  10                  15

Glu Glu Ala Ala Val Glu Glu Gln Glu Glu Ala Ala Glu Glu Asp Ala
            20                  25                  30

Glu Ala Glu Ala Glu Thr Glu Glu Thr Arg Ala Glu Glu Asp Glu Glu
        35                  40                  45

Glu Glu Glu Ala Lys Glu Ala Glu Asp Gly Pro Met Glu Glu Ser Lys
    50                  55                  60

Pro Lys Pro Arg Ser Phe Met Pro Asn Leu Val Pro Pro Lys Ile Pro
65                  70                  75                  80

Asp Gly Glu Arg Val Asp Phe Asp Asp Ile His Arg Lys Arg Met Glu
                85                  90                  95

Lys Asp Leu Asn Glu Leu Gln Ala Leu Ile Glu Ala His Phe Glu Asn
            100                 105                 110

Arg Lys Lys Glu Glu Glu Glu Leu Val Ser Leu Lys Asp Arg Ile Glu
        115                 120                 125

Arg Arg Arg Ala Glu Arg Ala Glu Gln Gln Arg Ile Arg Asn Glu Arg
    130                 135                 140

Glu Lys Glu Arg Gln Asn Arg Leu Ala Glu Glu Arg Ala Arg Arg Glu
145                 150                 155                 160

Glu Glu Glu Asn Arg Arg Lys Ala Gln Asp Glu Ala Arg Lys Lys Lys
                165                 170                 175

Ala Leu Ser Asn Met Met His Phe Gly Gly Tyr Ile Gln Lys Gln Ala
            180                 185                 190

Gln Thr Glu Arg Lys Ser Gly Lys Arg Gln Thr Glu Arg Glu Lys Lys
        195                 200                 205

Lys Lys Ile Leu Ala Glu Arg Arg Lys Val Leu Ala Ile Asp His Leu
    210                 215                 220

Asn Glu Asp Gln Leu Arg Glu Lys Ala Lys Glu Leu Trp Gln Ser Ile
225                 230                 235                 240

Tyr Asn Leu Glu Ala Glu Lys Phe Asp Leu Gln Glu Lys Phe Lys Gln
                245                 250                 255

Gln Lys Tyr Glu Ile Asn Val Leu Arg Asn Arg Ile Asn Asp Asn Gln
            260                 265                 270
```

```
Lys Val Ser Lys Thr Arg Gly Lys Ala Lys Val Thr Gly Arg Trp Lys
        275                 280                 285
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 495 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..486

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG GAT GAT ATC TAC AAG GCT GCG GTA GAG CAG CTG ACA GAA GAG CAG        48
Met Asp Asp Ile Tyr Lys Ala Ala Val Glu Gln Leu Thr Glu Glu Gln
  1               5                  10                  15

AAA AAT GAG TTC AAG GCA GCC TTC GAC ATC TTC GTG CTG GGC GCT GAG        96
Lys Asn Glu Phe Lys Ala Ala Phe Asp Ile Phe Val Leu Gly Ala Glu
            20                  25                  30

GAT GGC TGC ATC AGC ACC AAG GAG CTG GGC AAG GTG ATG AGG ATG CTG       144
Asp Gly Cys Ile Ser Thr Lys Glu Leu Gly Lys Val Met Arg Met Leu
        35                  40                  45

GGC CAG AAC CCC ACC CCT GAG GAG CTG CAG GAG ATG ATC GAT GAG GTG       192
Gly Gln Asn Pro Thr Pro Glu Glu Leu Gln Glu Met Ile Asp Glu Val
    50                  55                  60

GAC GAG GAC GGC AGC GGC ACG GTG GAC TTT GAT GAG TTC CTG GTC ATG       240
Asp Glu Asp Gly Ser Gly Thr Val Asp Phe Asp Glu Phe Leu Val Met
65                  70                  75                  80

ATG GTT CGG TGC ATG AAG GAC GAC AGC AAA GGG AAA TCT GAG GAG GAG       288
Met Val Arg Cys Met Lys Asp Asp Ser Lys Gly Lys Ser Glu Glu Glu
                85                  90                  95

CTG TCT GAC CTC TTC CGC ATG TTT GAC AAA AAT GCT GAT GGC TAC ATC       336
Leu Ser Asp Leu Phe Arg Met Phe Asp Lys Asn Ala Asp Gly Tyr Ile
            100                 105                 110

GAC CTG GAT GAG CTG AAG ATA ATG CTG CAG GCT ACA GGC GAG ACC ATC       384
Asp Leu Asp Glu Leu Lys Ile Met Leu Gln Ala Thr Gly Glu Thr Ile
        115                 120                 125

ACG GAG GAC GAC GAC ATC GAG GAG CTC ATG AAG GAC GGA GAC AAG AAC       432
Thr Glu Asp Asp Asp Ile Glu Glu Leu Met Lys Asp Gly Asp Lys Asn
    130                 135                 140
```

```
AAC GAC GGC CGC ATC GAC TAT GAT GAG TTC CTG GAG TTC ATG AAG GGG      480
Asn Asp Gly Arg Ile Asp Tyr Asp Glu Phe Leu Glu Phe Met Lys Gly
145                 150                 155                 160

GTC GAG TAAGGATCC                                                  495
Val Glu
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 162 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Met Asp Asp Ile Tyr Lys Ala Ala Val Glu Gln Leu Thr Glu Glu Gln
  1               5                  10                  15

Lys Asn Glu Phe Lys Ala Ala Phe Asp Ile Phe Val Leu Gly Ala Glu
             20                  25                  30

Asp Gly Cys Ile Ser Thr Lys Glu Leu Gly Lys Val Met Arg Met Leu
         35                  40                  45

Gly Gln Asn Pro Thr Pro Glu Glu Leu Gln Glu Met Ile Asp Glu Val
     50                  55                  60

Asp Glu Asp Gly Ser Gly Thr Val Asp Phe Asp Glu Phe Leu Val Met
 65                  70                  75                  80

Met Val Arg Cys Met Lys Asp Asp Ser Lys Gly Lys Ser Glu Glu Glu
                 85                  90                  95

Leu Ser Asp Leu Phe Arg Met Phe Asp Lys Asn Ala Asp Gly Tyr Ile
            100                 105                 110

Asp Leu Asp Glu Leu Lys Ile Met Leu Gln Ala Thr Gly Glu Thr Ile
        115                 120                 125

Thr Glu Asp Asp Asp Ile Glu Glu Leu Met Lys Asp Gly Asp Lys Asn
    130                 135                 140

Asn Asp Gly Arg Ile Asp Tyr Asp Glu Phe Leu Glu Phe Met Lys Gly
145                 150                 155                 160

Val Glu
```

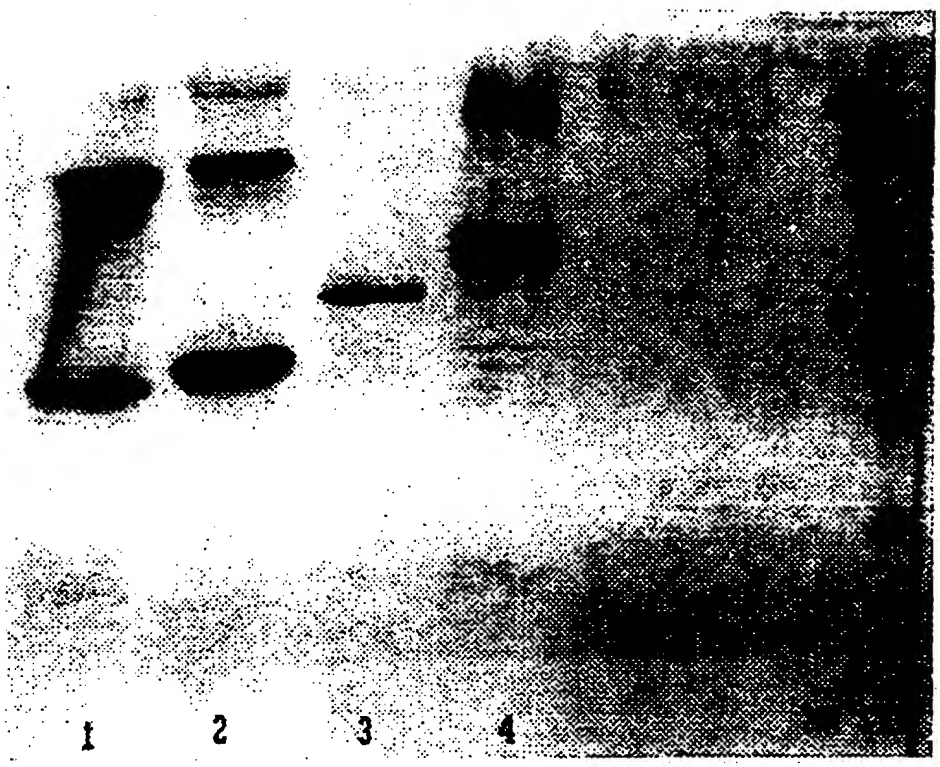


FIG. 1


FIG. 2

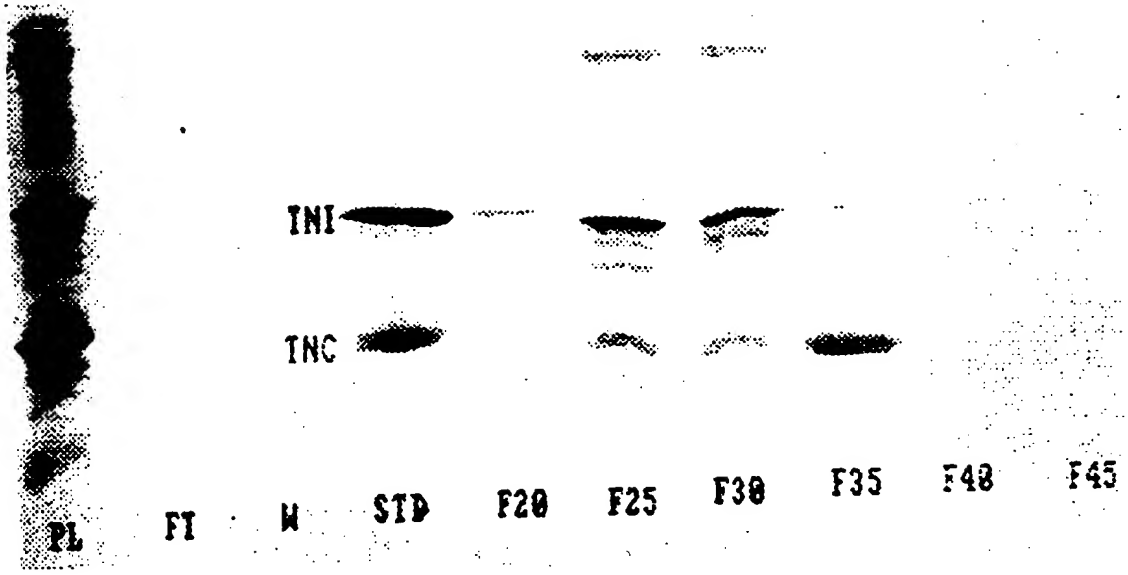


FIG. 3

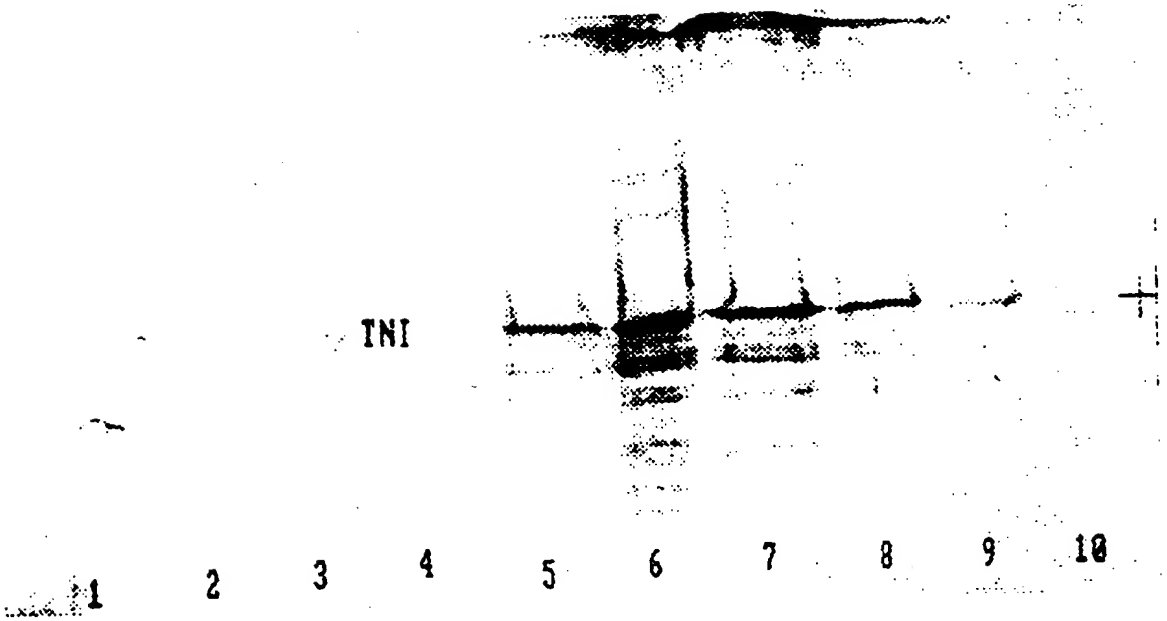


FIG. 4

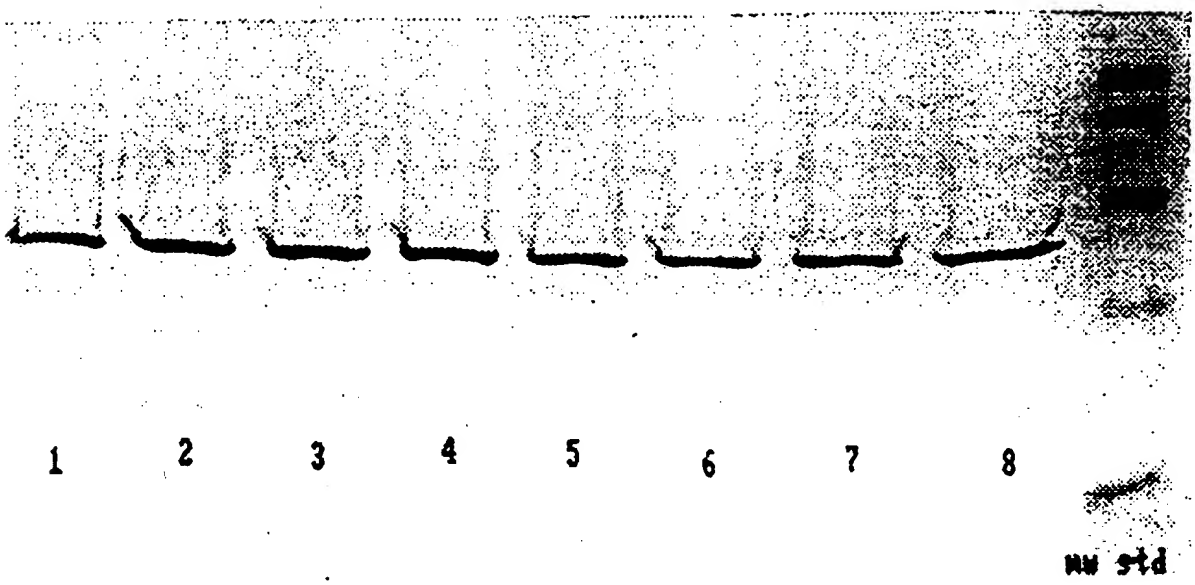


FIG. 5

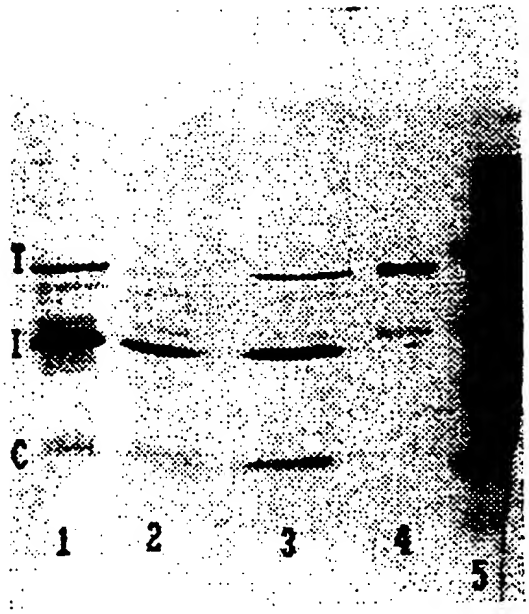


FIG. 6

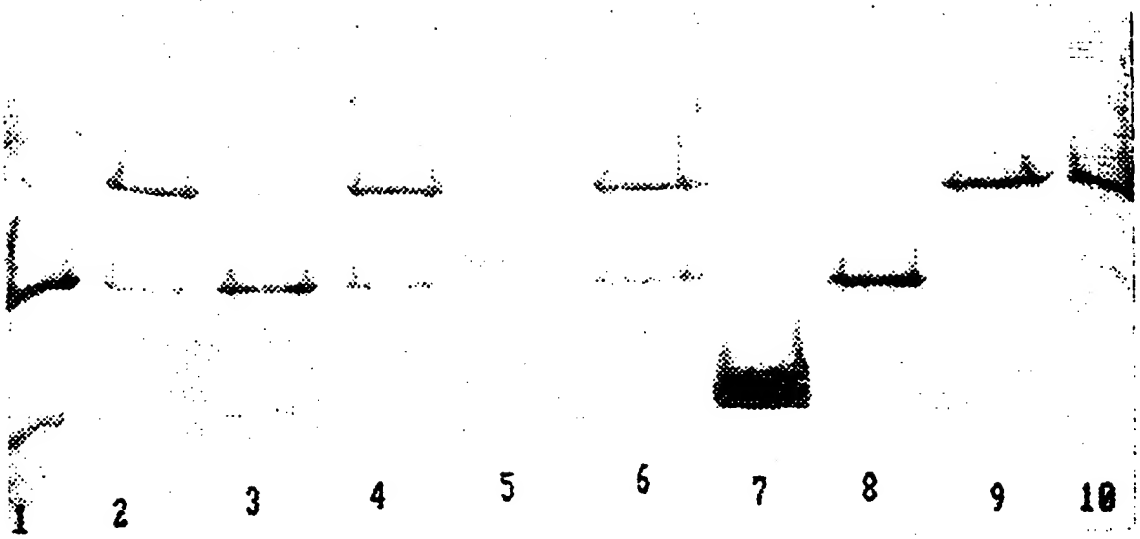


FIG. 7

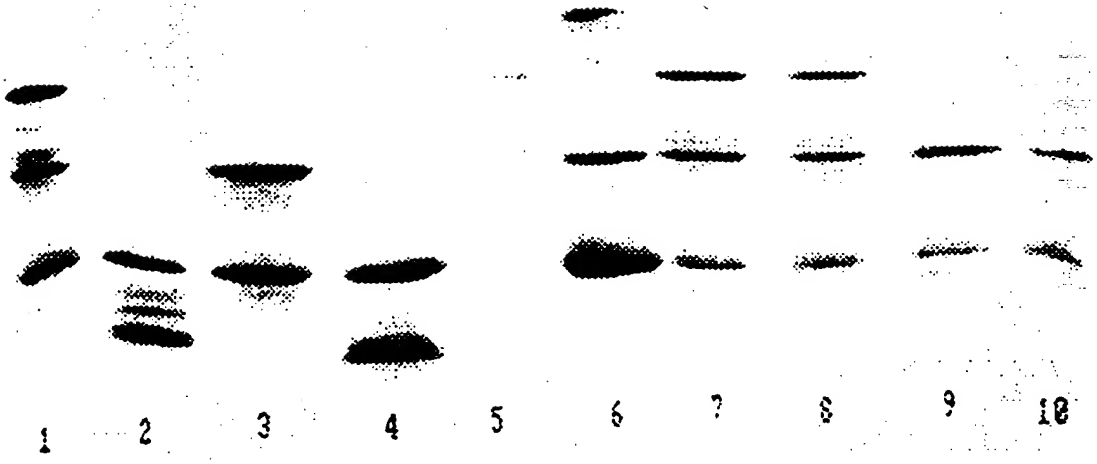


FIG. 8

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/06147

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K14/47 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | GB 2 275 774 A (PASTEUR SANOFI DIAGNOSTICS) 7 September 1994<br>cited in the application<br>see the whole document<br>see claims 4,5<br>see page 3, line 4-19 | 1-14, 25-28,31 |
| X | MOLECULAR IMMUNOLOGY, vol. 29, no. 2, 1 January 1992, pages 271-278, XP000400710<br>LARUE C ET AL: "NEW MONOCLONAL ANTIBODIES AS PROBES FOR HUMAN CARDIAC TROPONIN I: EPITOPIC ANALYSIS WITH SYNTHETIC PEPTIDES"<br>see page 1, column 2, line 20 - page 2, column 1, line 5 | 17 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 7 August 1997 | 18.08.97 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Cervigni, S |

Form PCT/ISA/210 (second sheet) (July 1992)

Inte. ional Application No
PCT/US 97/06147

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 95 12672 A (MEDICAL RES COUNCIL ;NERI DARIO (GB); WINTER GREGORY PAUL (GB); LA) 11 May 1995<br>see claim 9; example 10<br>--- | 19 |
| A | JOURNAL OF BIOCHEMISTRY,<br>vol. 117, no. 1, January 1995,<br>pages 158-161, XP002028679<br>OJIMA T ET AL: "AMINO ACID SEQUENCE OF C-TERMINAL 17 KDA CNBR-FRAGMENT OF AKAZARA SCALLOP TROPONIN-I"<br>see the whole document<br>see page 1, column 2, line 1-3<br>----- | |

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| GB 2275774 A | 07-09-94 | FR 2701954 A | 02-09-94 |
| | | CA 2116066 A | 24-08-94 |
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